GENERATING VIRUS-RESISTANT TRANSGENIC PLANTS HARBOURING ARTIFICIAL MICRORNA CONTAINING COMBINED SEQUENCES FROM TWO ORCHID VIRUSES

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NATIONAL UNIVERSITY OF SINGAPORE

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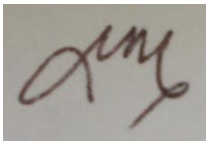
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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Summary

Orchid flowers have alluring morphology with diverse shapes and size and they are often used for ornamental purposes. The attractive orchid flowers quality will be greatly affected when orchids are infected with virus. There are two prevalent orchid-infecting viruses, namely *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV). CymMV and ORSV can infect almost every genus of orchids like *Cattleya, Cymbidium, Dendrobium, Phalaenopsis* and *Oncidium*. When orchids are infected with viruses, colour breaking will be seen on the flowers and in turn affect the attractiveness of the flowers. Preventing or curbing the spread of virus does not cure the root of the problem as long as orchids do not have the ability to resist against virus infection. Advances in genetic engineering and plant transformation development enable the generating of transgenic plants that have the capability to resist viral infection.

In this project, artificial microRNAs (amiRNAs) strategy was adopted to target CymMV and ORSV simultaneously along with *Agrobacterium*-mediated transformation and particle bombardment were employed to generate transgenic *N. benthamiana* and *Dendrobium* Chao Praya Smile that can resist against CymMV and/or ORSV infection. Orchids are slow growing and it will take years to obtain transgenic orchids that can resist against virus infection. *Nicotiana benthamiana* is a common host plant for CymMV and ORSV, and it grows faster than orchids, thus, transgenic *N. benthamiana* was generated first to test the feasibility of the amiRNAs strategy. AmiRNAs based on RNA-dependent RNA Polymerase of CymMV and ORSV were

designed and constructed into a single transgene, named amiR-CymMV-ORSV, in pGreen vector. Using *Agrobacterium*-mediated transformation, leaf explants of *N. benthamiana* was used, coupled with plant tissue culture techniques, to finally obtain T_2 transgenic *N. benthamiana* plants harbouring amiR-CymMV-ORSV. The transgene had successfully integrated into the plant genome and was able to express in the plant cell. *In vitro* transcripts of CymMV and ORSV were used as virus source to test the virus resistance ability of the T_2 transgenic *N. benthamiana*. From the results obtained, the T_2 transgenic *N. benthamiana* plants did not show any typical virus disease symptoms 21 days post inoculation and absence of viral coat protein in the plant cell. This is the first report on the transgenic *N. benthamiana* plants that can resist against CymMV and ORSV concurrently.

Attempts were made to generate transgenic *Dendrobium* Chao Praya Smile by using protocorms through *Agrobacterium*-mediated transformation. Due to the slow growth of orchid, only some putative transgenic orchids were obtained and tested positive by polymerase chain reaction (PCR). Nevertheless, the remaining putative transgenic orchids are still surviving on selection medium and this is an indication that the transgenic orchids could be produce and thus may possess the ability to resist against CymMV and ORSV infection.

The suitability of pollinia from *Dendrobium* Chao Praya Smile as starting materials in orchid transformation was investigated through *Agrobacterium*-mediated transformation and particle bombardment. The preliminary results suggested that there is potential of using pollinia as starting materials in orchid transformation.

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List of Abbreviations

Virus name

CymMV	*Cymbidium mosaic virus*
EBV	*Epstein-Barr virus*
ORSV	*Odontogloussum ringspot virus*
PVX	*Potato virus X*
PVY	*Potato virus Y*
TMV	*Tobacco mosaic virus*
TuMV	*Turnip mosaic virus*
TYMV	*Turnip yellow mosaic virus*

Chemical and reagents

MES	2-(*N*-morpholino)ethanesulfonic acid
AS	Acetosyringone
BA	6-benzyladenine (benzylaminopurine)
$CaCl_2$	Calcium chloride
Carbenicillin	Carbenicillin disodium
CTAB	Cetrimonium bromide
DIG	Digoxigenin
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
Kanamycin	Kanamycin monosulphate
KC medium	Knudson C medium
KCl	Potassium chloride
KH_2PO_4	Potassium di-hydrogen phosphate
LB medium	Luria-Bertani medium
MSO	L-methionine sulfoximine
$MgCl_2$	Magnesium chloride
MS medium	Murashige and Skoog medium
Na_2HPO_4	Disodium hydrogen phosphate
NAA	1-naphthaleneacetic acid
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NBT/BCIP	Nitro-blue-tetrazolium/5-bromo-4-chloro-3-
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulphate
SSC	Saline-sodium citrate
TAE buffer	Tris-acetate electrophoresis buffer
TE buffer	Tris-EDTA buffer
Tris	Trisaminomethane

Units and measurements

bp	base pairs(s)
cm	centimeter(s)
°C	degree Celsius
g	gram(s)
g/L	gram(s) per litre
h	hour(s)
kb	kilo base pair(s)
kDa	kilo dalton
L	litre(s)
m	meter(s)
M	moles(s) per litre
µg	microgram(s)
µL	microliter(s)
µM	micromolar
mg	milligram(s)
mg/L	milligram(s) per litre
min	minute(s)
mm	millimeter(s)
mM	millimole(s) per litre
nm	nanometer(s)
nt	nucleotide
OD	optical density
%	percentage
pH	potential of hydrogen
psi	pound(s) per square inch
× g	relative centrifugal force
rpm	revolutions per minute
sec	second(s)
U	unit(s)
w/v	Weight per volume
v/v	volume per volume

Others

amiRNA	artificial microRNA
bar	bialaphos resistance gene
CaMV	cauliflower mosaic virus
CP	coat protein
CP-MR	coat protein-mediated resistance
cDNA	complementary deoxyribonucleic acid
2x35S promoter	double cauliflower mosaic virus 35S coat protein
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
GUS	beta-glucuronidase
hpt	hygromycin phosphotransferase
miRNA	microRNA
mRNA	messenger ribonucleic acid

MP	movement protein
npt	neomycin phosphotransferase
ORF	opening reading frame
PAT	phsphophinothricin acetyl transferase
PCR	polymerase chain reaction
PTGS	post transcriptional gene silencing
PLB	protocorms-like body
PPT	phosphinothricin
RdRp	RNA-dependent RNA polymerase
RNAi	RNA interference
RISC	RNA-induced silencing complex
RT	reverse transcription
RNA	ribonucleotide
RNase	ribonuclease
T-DNA	transfer deoxyribonucleic acid region
TGB	triple gene block
Ti	tumour-inducing plasmid
Vir	virulence gene
UV	ultraviolet
UTR	untranslated region
VIGS	virus-induced gene silencing
Vis	visible light
wt	wild-type

Chapter 1 Introduction

1.1 Orchids

Orchids are one of the largest and diverse flowering plants families and are members under the Orchidaceae family (Yu and Xu, 2007). They are highly adaptive and can be found in the tropical and subtropical regions of Asia, and in the mountain of Central and South America. Therefore, they are highly diversified in terms of morphological and physiological characteristics. This contributes to the presence of more than 800 described genera, 25,000 identified species and 120,000 cultivated hybrids throughout the world (Arditti, 1992; Chan et al., 2006a; 2006b).

Orchid flowers have unique appearance as comparing to other angiosperms' blossoms. Pollinia are a coherent mass of pollen grains for effective pollination as a single unit. They are located near the column or gynostermium, a fusion of stamen, styles and stigmas. The labellum is located opposite to the column and varies in structure and visually striking to attract potential pollinators. The sepals of orchids are usually developed as petaloid.

Among the genera, *Dendrobium* is the second largest genus in the Orchidaceae family. *Dendrobium* orchids have a sympodial growth pattern and are found in different growing habits, as lithophytic or epiphytic (Baker and Baker, 1996). It produces lateral inflorescences near the top of the stem with attractive flowers (Sim, 1996). *Dendrobium* flowers vary in size, and colour. They are recognised by two pairs of pollinia without caudicles. The fusion of the lateral

sepals' wide base, the labellum and the column-foot form the spur-like mentum. The column-foot is connected to the base of the labellum. The sepals are narrower than the petals though they resemble each other (Figure 1.1).

Orchids are valued for their cut-flowers and potted plants on the global market due to their exquisite beauty and diversified patterns. Orchid cut-flower industry has emerged as a strong demand export item and has been contributing towards the economics for countries such as Singapore, Thailand, Taiwan and other South-East Asian countries. Orchids are significant in Singapore as *Vanda* Miss Joaquim, a hybrid orchid species (Soon, 1982), is the national flower.

With increasing demands from the consumers, orchid species with flowers of unique shapes, vibrant colours, patterns, long vase-life, fragrance and traits likes resistance against pests and disease are economically extremely desirable for floriculturists (Chan et al., 2006a). To produce these desired agronomic orchid traits with commercial values, conventional plant breeding methods, through sexual hybridisation and selection of orchid breeds and polyploids were used. However, this approach is difficult, time-consuming and relies on natural resources (Anzai et al., 1996). Furthermore, due to the genetic background of the same or closely related species, the gene pool available for new traits is restricted. To meet the supply and demands of the orchid industry, modern gene transformation techniques are available as a practical alternative to manipulate desired characteristics in orchids (Tee et al., 2003; Teixeira da Silva, 2004; Tanaka et al., 2005a; Chan et al., 2006b).

Figure 1.1 *Dendrobium* Chao Praya Smile.

The white with purplish traces flower of *Dendrobium* Chao Praya Smile, the orchid used in this project.

1.2 Plant virus

Koch postulated that an infectious agent must be able to associate and induce symptoms on its infected hosts (Koch, 1893). In addition, it must be able to isolate from the infected host and cultured onto a medium. The culture is then able to cause same or similar symptoms on another infected plant of the same host and able to be recovered again (Koch, 1893; Kung and Yang, 1998).

Plants are unable to grow normally when they are infected by undesirable growth environments or plant pathogens. Undesirable environmental conditions include drought, flood, nutrients deficient or over-dose of herbicide and pollution would cause physiological stress to the plants. Plant pathogens such as oomycetes, bacteria, phytoplasma, nematodes, parasitic plants, fungi and viruses will utilise plants for its own survival.

Tobacco mosaic virus (TMV) was the first virus to be discovered in the late 19th century and first purified by Wendell M. Stanley (Zaitlin, 1998). Plant virus induces disease symptoms with developmental abnormalities caused significant losses to the agricultural and horticultural industries. Those developmental abnormalities are flower distortion, leaf curling, stunting; chlorosis; and necrosis. These symptoms require virus-host interaction which in turn affects the plant physiology (Culver and Padmanabhan, 2007). The virus-host interaction will affect virus replication, cell-to-cell or systemic movement, suppression or initiate host defense system (Carrington et al., 1996; Whitham et al., 2006). Plant viruses begin replication at the site of infection by entering into host plant cells by mechanical injury (Huijberts et al., 1990)

vectors such as insects or fungal parasites (Brown et al., 1995; Campbell, 1996) or grafting (Damsteegt et al., 2004). Replication starts in the initially infected cells and adjacent cells via cell-to-cell movement involving movement proteins (MP) (Vanlent et al., 1991; Lucas, 2006). Plasmodesmata are involved in cell trafficking and allow adjacent cells to communicate and thus, they are necessary for plant growth and development and also for defense against pathogen attacks. Therefore, plamodesmata are the sites of target for virus to begin its infection (Wolf et al., 1989; Wolf et al., 1991; Lucas and Gilbertson, 1994). Virus will then spread to the vascular system and begin systemic infection and sometimes, additional protein such as coat protein (CP) of viruses would be required (Heaton et al., 1991). During the course of virus infection, the plants will recognise CP as an early warning towards pathogen invasion (Callaway et al., 2001). This would let the plant cells to adjust its cell program and activate the host defense system to face the invading pathogens (Nimchuk et al., 2003). A hypersensitive response is pathogen-induced host defense system upon activation of defense gene expression by host resistance gene products (Kanchroo et al., 2006). Programmed cell death at the site of infection would be the choice made by plant cells in an attempt to block the spread of virus infection (Baker et al., 1997).

1.2.1 Orchid infecting viruses

However, when these ornamental orchids are infected with viruses, the growth and development of orchids will be retarded. Furthermore, the quality and quantity of orchid flowers will be compromised. Among the over 50 orchid-infecting viruses, there are two most prevalent and

economically important viruses, CymMV and ORSV (Zettler et al., 1990). CymMV and ORSV are economically important as they can be found world- (Jensen and Gold, 1951) . According to Wong et al. (1994), incidence rate of orchids found to be infected with CymMV was higher than with ORSV (Wong et al., 1994). CymMV and ORSV can be transmitted mechanically via inoculation of infected sap; or through contaminated equipment such as cutting tools. These viruses are not transmitted by seeds or vectors (Francki, 1970; Namba and Ishii, 1971; Wisler et al., 1986). CymMV induces chlorotic or necrotic patches on the leaves and the flowers of orchids. Furthermore, flowers infected with CymMV are deformed and displayed colour breaking. As for ORSV, it causes streak or striped mosaic, diamond mottle or ringspots on orchid leaves and it often results in ringspots or colour breaking on infected flowers (Zettler et al., 1990; Wong et al., 1994). Other than its natural hosts, CymMV and ORSV are able to infect systemically other plant species including *N. benthamiana* which is a Solanaceous plant that is a common systemic host for CymMV and ORSV. For *N. benthamiana* infected by CymMV, White intermittent lines can be observed on the leaves of CymMV-infected *N. benthamiana*. Mild mosaics on leaves and emerging distorted leaves are usual symptoms by ORSV in *N. benthamiana*.

1.2.1.1 *Cymbidium mosaic virus*

CymMV belongs to the potexvirus group of virus. Viruses within this group are normally flexouous and filamentous with the particles of

450-550 (Francki, 1970). CymMV has a 5'-terminus capped single-stranded positive sense RNA genome, 6227 nucleotide (nt) in length, excluding the polyadenylated at the 3'-terminus. The genome of CymMV consists of five open reading frames (ORF) namely RNA-dependent RNA polymerase (RdRp) gene, triple gene block (TGB) and coat protein (CP) gene. The RdRp gene (nt 73-4326) will produce a 160 kilo Dalton (kDa) protein comprises of three conserved domains: a methyltransferase domain (nt 73-975), a putative NTP-binding domain (nt 2049-2583) and a core binding domain (nt 3355-4101) (Wong et al., 1997).

Between RdRp and TGB, there is a six-nt intergenic region. TGB (nt 4333-5478) is considered to be the movement protein (MP) with three overlapping ORFs that encode three proteins of 26 kDa, 13 kDa and 10 kDa respectively (Wong et al., 1997). TGB 1 (nt 4333-5022) contains a NTPbinding helicase motif (nt 4420-4446). TGB 2 gene (nt 5115-5189) has a consensus sequence of PXXGDXXHXXPSGGXYXDGTKXXXY which can be found in other potexviruses and the carlaviruses. TGB 3 has a high level of variability (Wong et al., 1997). The CP gene (nt 5480- 6152) encodes CP needed for encapsidation of the viral RNA. The N-terminal of CP displays high level of variability and therefore produces low serological cross-reactivity in potexviruses (Chia et al., 1992).

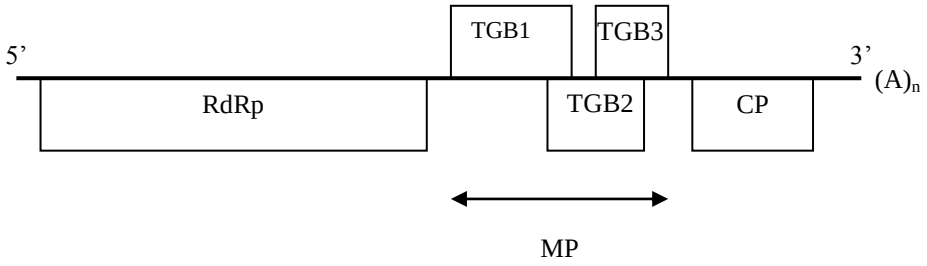


Figure 1.2 Genome organisation of *Cymbidium mosaic virus*.

CymMV has a 5’-terminus capped single-stranded positive sense RNA genome, 6227 nucleotide (nt) in length, excluding the polyadenylated at the 3’-terminus. The genome of CymMV consists of five open reading frames (ORF) namely RNA-dependent RNA polymerase gene (nt 73-4326), triple gene block (nt 4333-5478) and coat protein gene (nt 5480- 6152).

1.2.1.2 *Odontoglossum ringspot virus*

ORSV belongs to the tobamovirus group. The virus particles are rigid rod-shaped of approximately 18 × 300 nm with a 4 nm in diameter central hollow (Webster and Granoff, 1994). ORSV has a 5'-terminus capped single-stranded positive sense RNA genomics of 6609 nt long (Chng et al., 1996) with non-polyadenylated 3'-terminus. The 5'-untranslated region (UTR) is of 62 nt long and consists of three copies of ACAATTAC direct repeats and eight copies of CAA or ACA triplets in the region. The 412 nt 3'-UTR is characterized by a tRNA-like structure and three consecutive homologous regions (Chng et al., 1996). The ORSV genome encodes four genes: 126/183 kDa RdRp gene, MP gene and CP gene. Both 126 kDa and 183 kDa proteins of RdRp are translated from the same ORF (nt 63-3401/-3490), with 183kDa protein being produced as a readthrough of a leaky amber stop codon of the 126 kDa protein at nt 3399. There are three functional domains that are being identified in the RdRp. The first domain is a putative methyltransferase required for cap formation (Habili and Symons, 1989). It has four different conserved motifs (Alonso et al., 1991) located at aa 72-287 (Chng et al., 1996) and may contribute to the methyltransferase activity. The helicase is the second domain at aa 820-1074 (Chng et al., 1996) containing six conserved motifs (I-VI) (Evans et al., 1985; Goorbalenya and Koonin, 1989; Habili and Symons, 1989). The third domain is the polymerase domain defined by a GDD consensus

sequence with four conserved motif at aa 1372-1503 (Chng et al., 1996). The MP gene (nt 4807-5718) overlaps with the 3'-terminus of RdRp by 94 nt and encodes a 33 kDa MP required for cell-to-cell movement of the virus (Ryu and Park, 1995). Within the MP gene, there is a putative origin of assembly whose secondary structure has been determined to have two loops and a XXGrepeat motif. The structure of the origin of assembly is necessary for binding and initiation of assembly of the coat protein (Turner et al., 1988; Chng et al., 1996). ORSV CP gene (nt 5721-6197) begins two nt downstream of the MP. CP gene has three highly conserved RNA-binding motifs (Chng et al., 1996).

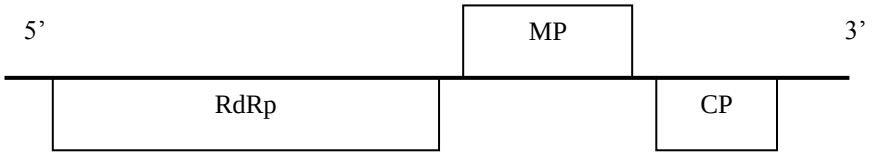


Figure 1.3 Genome organisation of *Odontoglossum ringspot virus*.

ORSV has a 5’-terminus capped single-stranded positive sense RNA genomics of 6609 nt long with non-polyadenylated 3’-terminus. The ORSV genome encodes four genes: 126/183 kDa RdRp gene (nt 63-3401/-3490), MP gene (nt 4807-5718) and CP gene (nt 5721-6197).

1.2.2 Synergism between plant viruses

One or more different viruses can infected the plants at the same period and the doubly or multiply-infected plants could result in intensification of disease symptoms. The intensified disease symptoms could be accompanied by the higher amount of one or both viruses in the infected plants and such phenomenon is termed synergism.

Synergism was observed in CymMV and ORSV-infected plants where the new grown leaves were severely distorted with dark-green islands (Ajjikuttira, 2003) and this was in line with previous reports (Lawson and Brannigan, 1986; Pearson and Cole, 1991). Ajjikuttira (2003) speculated that the CP CymMV could lead to the accumulation of ORSV RNA level resulting in the disease symptoms intensification.

1.3 Plant transformation

There is significant advancement in plant genetic transformation techniques in the last two decades (Tanaka et al., 2005b; Vain, 2006). These allow the introduction and stable expression of foreign genes into plant genome. The plant cells are totipotent and in general have the capability to regenerate into whole fertile plants. In the mid-1970s, crop plants such as corns, canola, soybeans and tomatoes with genetically modified traits were produced using developing transgenic techniques. From 1980s, the transgenic techniques have matured and transgenic agricultural crops like barley, maize, rice and wheat with specific traits were generated. Most of these transgenic plants were produced using either *Agrobacterium*-mediated transformation or particle bombardment (Khentry et al., 2007). Due to the long vegetative state of orchids, molecular analysis of T_1 progeny is time-consuming. Therefore in orchids, only T_0 transgenic lines were used for validation by Northern, Southern and Western blot analyses for the presence, copy number, transcriptional activity of foreign gene incorporated (Liau et al., 2003a).

Compared with the growing number of reports of genetically engineered crops in the literature available, very few studies have demonstrated engineered plants for orchids. The transgenic orchids were created using genetic transformation coupled with tissue culture methods. To obtain reproducible genetic transformation systems in orchids, there are some essential factors to be considered. These factors are starting materials for transformation, methods of transformation, and suitable markers to select transformants. These factors will cumulatively affect the transformation efficiency. Therefore, it is a need

to deliberate and optimise these factors for a reproducible and efficient genetic transformation system in orchids.

1.3.1 Starting materials

In order to achieve reliable orchid transformation with maximal efficiency, the choice of starting materials is one of the important factors. During transformation and regeneration process, the totipotency of plant cells, or its regeneration capacity is affected by factors such as cell division cycle, competence of the cells around wound sites and the amount of phenolic compound produced by defense mechanism. Using plant tissue culture techniques, orchids were able to successfully propagate in mass as horticultural crops (Belarmino and Mii, 2000). They used *Phalaenopsis* cell clumps as starting materials for transformation. However, starting materials of meristematic tissue with great regeneration capacity like calli, protocorms-like bodies (PLBs), protocorms are more commonly used in orchid transformation.

Calli are groups of actively dividing uniform cells that can play a role for the integration of foreign genes into the orchid genome through transformation to obtain stable transformants (Binns and Thomashow, 1988). Furthermore, when transformed callus begins organogenesis in the selection media, this process prevents the possibility of regenerating chimeric transgenic orchids. Hence, there were reports of using calli to produce transgenic orchids (Men et al., 2003b; Tee and Maziah, 2005; Sjahril and Mii, 2006; Chai et al., 2007).

PLBs originated from single somatic cells and thus presumed to be genetically uniform (Park et al., 1996; Park et al., 2002). PLBs can be easily induced from various somatic tissues such as young leaves, stem segments, flower stalks and roots, regardless of cultivar. Meristematic cells of PLBs in early development stages are actively dividing which increase its regeneration capacity to differentiate into shoot after transformation (Kuehnle and Sugii, 1992; Park et al., 2002; Liau et al., 2003a; 2003b). Taken all these properties of PLBs, there were successful transformation using *Dendrobium*, *Oncidium* and *Phalaenopsis* (Chia et al., 1994; Nan et al., 1998; Yang et al., 1999; Knapp et al., 2000; Chai et al., 2002; Liau et al., 2003b; Men et al., 2003a; Men et al., 2003b; You et al., 2003; Liao et al., 2004; Chan et al., 2005b; Sjahril and Mii, 2006; Chin et al., 2007; Yee et al., 2008). Protocorms have the same properties as PLBs but they derived from seed germination (Kuehnle and Sugii, 1992; Yu et al., 1999; Knapp et al., 2000; Chang et al., 2005; Mishiba et al., 2005; Suwanaketchanatit et al., 2007).

1.3.2 Transformation methods

With advancement in orchid transformation system, there are many transformation methods available and they can be broadly classified into three groups: germ line-mediated gene transformation, direct DNA delivery and vector-mediated gene transformation.

Germ line-mediated gene transformation methods include infiltration and injection of ovary and embryo sac and pollen-tube pathway. As for direct DNA delivery, there are many approaches, namely electroporation, laser

microbeam, lipofection transfection, microinjection, particle bombardment, PEG-mediated transformation and protoplast transformation. *Agrobacterium*-mediated and virus-mediated transformation are categorised as vector-mediated gene transformation. When it comes to orchid transformation, *Agrobacterium*-mediated transformation and particle bombardment are the most popular methods.

1.3.2.1 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is a soil bacterium that carries "tumour-inducing" (Ti) plasmid which in turn contains a transferred DNA (T-DNA) region. Agrobacteria contains virulence (vir) genes to help in the transfer of T-DNA region. Vir proteins produced will excise the T-DNA region from the plasmid and transported the T-DNA region into the host plant cell. Furthermore, vir proteins can increase the permeability of the host plant cells to allow the T-DNA region to enter smoothly along with other vir proteins. The vir proteins present in the host plant cell will guide the T-DNA into the nuclear and integrate into the host plant genome (Hooykaas and Schilperoort, 1992). Hence, Agrobacteria provide a natural-available gene transfer system to introduce and express DNA stably in different plant species (Chilton et al., 1977).

Agrobacterium-mediated transformation is well-established for plant transformation over the past decades. It has emerged as one of the popular as it provided several advantages. Firstly, *Agrobacterium*-mediated transformation is easy to manipulation and does not require

specialised materials or equipment and thus this keeps the cost of the method low. Second, there are fewer copies of T-DNA with defined border sequences integrated into the plant genome with minimal rearrangement. Third, there is preferential integration of the T-DNA region into transcriptionally active regions of the plant genome. This will lead to high transformation efficiency with high quality and fertility of transgenic plants (Tingay et al., 1997; Komari et al., 1998). High transformation efficiency by *Agrobacterium*-mediated transformation is partly dependent on the expression of vir genes. Vir genes are induced by phenolic compounds such as acetosyringone, coumaryl alcohol and sinapul alcohol. Dicotyledons produce phenolic compounds at the wound site and this allows the Agrobacteria to express the vir genes and promotes transfer of the T-DNA region in the host plant nuclear (Bolton et al., 1986). However, monocots generally do not produce phenolic compounds or at low level at the injured site (Usami et al., 1987; Binns, 1990). Thus, monocots such as orchids could not achieve high and reproducible transformation and regeneration by *Agrobacterium*-mediated transformation (Gelvin and Liu, 1994; Nan et al., 1997; Men et al., 2003a). There are few reports on the success of *Agrobacterium*-mediated transformation on orchids until Nan et al. (1997) identified the presence of phenolic compound, coniferyl alcohol, in *Dendrobium* PLBs grown under light. They showed that these phenolic compounds were able to activate the vir genes in

Agrobacteria. Furthermore, there are reports on addition of exogenous phenolic compounds for maize and rice transformation (Hiei et al., 1994; Ishida et al., 1996), the feasibility of exogenous acetosyringone was achieved by Hsieh et al. (1997) in *Phalaenopsis* PLBs transformation. The authors added 100 µM of acetosyringone along with regeneration medium to the Agrobacteria culture before infection. The addition of acetosyringone helps to enhance the transformation events. Taken together these observations set the path towards orchids transformation using *Agrobacterium*-mediated transformation. Acetosyringone was added to the Agrobacteria culture and also to the medium for co-cultivation and enhanced transformation efficiency was achieved in *Cymbidium, Dendrobium*, *Onicidum* and *Phalaenopsis* transformation (Belarmino and Mii, 2000; Yu et al., 2001; Chai et al., 2002; Liau et al., 2003a; Men et al., 2003a; Chan et al., 2005b; Mishiba et al., 2005; Chin et al., 2007). The transgenic nature of the transgenic orchids generated was confirmed with PCR analysis and Southern blot and the results were reproducible (Liau et al., 2003a). On the other hand, only a few transgenic plants were obtained in the absence of acetosyringone.

The *Agrobacterium* strain and Ti plasmid used may influence the orchid transformation efficiency too. There is a selection of *A. tumefaciens* strains available and some examples are EHA101 (pEKH-WT) (Sjahril et al., 2006), EHA101 (pIG121Hm) (Mishiba et al., 2005), EHA105 (pMT1) (Hsieh et al., 1997), LBA4301 (Nan et

al., 1997), LBA4404 (pTOK233) (Yu et al., 2001; Chai et al., 2002). Men et al. (2003) observed a high proportion of GUS staining in AGL-infected than EHA105-infected *Dendrobium* PLBs (Men et al., 2003b). On the other hand, Liau et al. (2003b) did not found any difference in transformation efficiency between EHA105-infected and LBA4404-infected name of orchids PLBs. However, they observed a higher shoot formation rate in EHA-transformed tissues than LBA4404. This was due to the overgrowth of LBA4404 even though same antibiotics were used to cull EHA105 and LBA4404. On the other hands, others have suggested that the success of transformation efficiency is not influenced by the Agrobacterium strain alone (Hiei et al., 1997; Belarmino and Mii, 2000).

Foreign gene and selectable marker introduced into the target plant cells as a single binary vector produced more consistent results as compared to when they are introduced as separate vectors (cotransformation) (Yu et al., 2001; Liao et al., 2004; Chan et al., 2005b).

As *A. tumefaciens* was used for orchid transformation, it is necessary to add antibiotics to cull its growth and at the same time, selection agents must be present to screen for transformed tissues. The ideal antibiotic used must not affect the plant regeneration, does not negate the effect of plant growth regulators, and it should be effective in inhibiting the growth of Agrobacteria yet inexpensive (Cheng et al., 1998). The effects by different antibiotics in plant transformation

were demonstrated for several species (Hammerschlag et al., 1997). When it comes to orchid transformation, depending on the strain of Agrobacterium used, single or combination of antibiotics like timentin, cefotaxime, carbenicillin, meropenem can be used (Liau et al., 2003a; Mishiba et al., 2005; Sjahril and Mii, 2006).

Agrobacteria density is another factor to consider for successful orchid transformation. Even though Agrobacteria density was not fixed in transformation events, most reports used a density of OD600 of 0.2-1.0 to increase transformation efficiency (Hsieh et al., 1997; Chai et al., 2002; Liau et al., 2003b; You et al., 2003; Mishiba et al., 2005).

1.3.2.2 Particle bombardment

Not every orchid transformation can be conducted successfully using *Agrobacterium*-mediated transformation. The alternative popular approach is direct gene transformation via particle bombardment, also known as biolistic transformation or microprojectile bombardment. This plant transformation was introduced in 1987 by Klein et al. and the authors demonstrated it is an efficient method for foreign genes to integrate stably into the target plant genome (Klein et al., 1987).

In particle bombardment, plasmid DNA is coated onto the surface of microprojectiles and penetrated into the target plant cell under high-pressure blasts of helium gas (Klein et al., 1988). The DNA that

penetrated into the target plant cells may either integrate into the target plant genome at randomly location, or expressed transiently in the target plant cytoplasm, or degrade. Based on this system, a commercial available particle gun was marketed by Bio-Rad Laboratories, Inc. that uses high helium pressure blasts (Ye et al., 1990). The size and materials of microprojectiles, amount of plasmid DNA coated onto the microprojectiles and the gas pressure used are categorised under the physical parameters aspect of particle bombardment. These physical parameters can affect the level of penetration of plasmid DNA into the target plant cell and transformation efficiency. Beside physical parameters, biological parameters contribute the other half towards a successful transformation with decent transformation efficiency using particle bombardment (Christou, 1994; Nan and Kuehnle, 1995a). Biological parameters include choice of vectors with suitable promoters and selection markers, pre- and post-bombardment culture condition, the physiology state of the target plant tissue (Sanford et al., 1993).

Particle bombardment offers several advantages over other transformation methods. Firstly, it is not restricted to dicotyledons as it can be used to transform monocotyledons too (Klein et al., 1987). Secondly, the transformation efficiency does not dependent entirely on the biological parameters as the DNA delivery mechanism depends on the physical parameters. Nevertheless, to enhance the transformation efficiency, deliberation on the choice of explants'

properties like regeneration capability and possibility of chimeric plant generated should be conducted. Optimisation of the physical parameters should be performed to reduced cell damaged inflicted during the process. Lastly, stable and heritable generations of transgenic plants can be obtained. However multiple plasmids tends to clustered together via concatemerisation leading to integration of multiple copies into the target plant genome, and this will create undesirable consequences like transgene instability, silencing and rearrangements occurred at a higher rate, as compared to methods like *Agrobacterium*-mediated transformation (Birch, 1997).

The first reports introducing foreign genes into orchid by particle bombardment was by Chia et al. (1994) in *Vanda* and Kuehnle and Sugii (1992) in *Dendrobium* and subsequently successful gene transfer in *Brassia*, *Cattleya*, *Cymbidium*, *Dendrobium*, *Doritaenopsis*, *Oncidium*, and *Phalaenopsis* were reported (Nan and Kuehnle, 1995a; Anzai et al., 1996; Yang et al., 1999; Yu et al., 1999; Knapp et al., 2000; Men et al., 2003b; Su and Hsu, 2003; Liao et al., 2004; Chan et al., 2005b; Chang et al., 2005; Li et al., 2005; Tee and Maziah, 2005; Suwanaketchanatit et al., 2007).

Even though particle bombardment was used to transform various species of orchids with different starting materials, the transformation efficiency is low and foreign genes were not integrated into the target plant genome consistently (Yang et al., 1999; Yu et al., 1999; Men et al., 2003b). Furthermore, this approach

may generate chimeric transgenic plants. This happened as non-transformed cells were not removed effectively during each subculture stage.

1.3.2.3 Other transformation methods used

Other direct gene transformation methods namely electroinjection, electrophoresis, pollen tube pathway, and seed imbibition have been reported for transformation in *Calanthe*, *Dendrobium* and *Phalaenopsis* (Hsieh and Huang, 1995; Nan and Kuehnle, 1995b). These methods are not commonly used due to the low transformation efficiency. Amongst these methods, pollen tube pathway is frequently used; the foreign gene moving through the column formed by the pollen tubes to enter the embryo sac and transforming the eggs and zygotes inside. Pollen tube pathway had successfully transform *Dendrobium* and *Phalaenopsis* (Hsieh and Huang, 1995; Nan and Kuehnle, 1995b).

1.3.3 Selection makers

The plant transformation efficiency is not 100%, and it is even lower when it comes to orchid transformation. Orchid low transformation rate ranging from 1.5% to 3.4% was reported (Chai et al., 2002; Liau et al., 2003a; Men et al., 2003). This means that most of the foreign genes did not manage to enter the orchid cells and not all the introduced foreign genes are integrated into orchid genome. Furthermore, foreign genes that are incorporated into the orchid genome do not confer a phenotype for convenient selection of transformed orchid cells. Hence, a selectable

marker gene is coupled with the foreign gene and introduced into the target plant cell. The selectable marker gene will express a protein with a specific enzymatic activity. The presence of such proteins can either protect the transformed cells from death when a toxic agent is introduced into the culture medium or visually screen for transformed cells when a histochemical test is performed. This allows the selection of transformed cells from the nontransformed cells.

There are numerous selection systems available for plant transformation and the frequently used systems are antibiotic, herbicide, pathogen resistance, and visual selection markers (Miki and McHugh, 2004; Tian, 2006).

Antibiotics resistance is conferred by the presence of neomycin phosphotranserase or hygromycin phosphotransferase or encoded by NPT II and HPT II, respectively. These genes will inactivate the aminoglycoside antibiotics like genticin, hygromycin, kanamycin, neomycin and paromomycin and eliminate their toxicity by phosphorylation. Kanamycin is the most frequently used antibiotic to screen for transformed cells, however, some orchids are naturally resistant to it (Kuehnle and Sugii, 1992; Belarmino and Mii, 2000; Chin et al., 2007). The amount of kanamycin needed to screen for transformed orchid cells ranged from 100 to 200 mg/L, much higher than the minimal amount of 50 mg/L for normal usage as selective agent (Chia et al., 1994). The high concentration of kanamycin can inhibit the regeneration of putative transformed tissues if selection

needs a long period of time. Thus, it is not suitable for orchid transformation as untransformed orchid tissues need few months or longer to die off due to its slow and long vegetable growth.

Hygromycin is preferred over kanamycin as it is more toxic thus it can eliminate nontransformed cells more effectively at a lower concentration than kanamycin (Men et al., 2003b; Chang et al., 2005; Yee et al., 2008).

Herbicide resistance depends on the bialaphos resistance gene (bar) that encodes phosphinothricin N-acetyltransferase (PAT) (Block et al., 1989). PAT can repress phosphinothricin (PPT), an analogue to glutamate. If glutamate is not converted to glutamine by glutamate synthetase, toxic ammonia will accumulate and the plant cell will die due to dysfunction of chloroplast and photosynthesis (Lindsey, 1992). Transformed cells containing PAT can survive in culture medium supplemented with purified PPT. Purified PPT is preferred over herbicide. Even though herbicide contains PPT, non-specific growth regulators are also present in the herbicide, making them unsuitable for direct addition into the culture medium. Purified PPT was used for transformation in *Brassia*, *Cattleya*, and *Doritaenopsis* (Knapp et al., 2000). PPT can increase the cost of the experiments and fortunately, a cheaper alternative, L-methionine suloximine (MSO), is available (Maughan and Cobbett, 2003). MSO and PPT have similar structure and function and yet MSO is 40 times more effective and 10 times less

costly (Maughan and Cobbett, 2003). MSO had been used for selection of transgenic *Dendrobium* hybrids (Chai et al., 2007; Ding et al., 2013).

Orchids suffered soft rot disease caused by pathogen *Erwinia carotovora*. Transgenic *Phalaenopsis* and *Oncidium* with antimicrobial properties pepper ferredoxin-like protein gene showed to have enhanced resistance against *E. carotovora* infection (Liau et al., 2003b; You et al., 2003; Chan et al., 2005a). This approach does not have much inhibitory effort or toxicity as compared to usage of antibiotic selection and the selection period is shorter due to the usage of bacteria as selection agent.

Visual selection relies on the ability of the selectable marker to produce colur upon inspection by photon-counting video camera-photomultiplier system or histochemical test. The former system made use of luciferase gene expressed in transgenic tissues (Chia et al., 1994; Yang et al., 1999) but it required imaging facilities and labour-intensive manual selection and thus it is not used frequently. The latter test is more commonly used in plant transformation and it needs beta-glucuronidase (GUS) encoded by *gusA*. Detection of GUS presence can be easily performed by histochemical test and fluorigenic substrate and it was used in orchid transformation (Men et al., 2003b; Mishiba et al., 2005).

1.4 Strategies towards virus-resistant transgenic plants

1.4.1 Coat protein-mediated resistance

The first virus-derived resistant transgenic plant was demonstrated by Abel et al. (1986) where they used the CP gene of TMV (Abel et al., 1986).

Subsequently, Register and Beachy (1988) suggested CP-mediated resistance (CP-MR) against TMV was through the interference of disassembly of the challenged TMV virions. CP-MR describes the virus infection and disease development resistance in transgenic plants were caused by the expression and accumulation of virus CP (Beachy et al., 1990). CP-MR has several features, namely fewer infection sites on inoculated leaves (Loesch-Fries et al., 1987; Nelson et al., 1987; Hemenway et al., 1988), reduced rate in systemic disease development (Nelson et al., 1987; Tumer et al., 1987; van Dun et al., 1987; Cuozzo et al., 1988; Hemenway et al., 1988; Van Dun and Bol, 1988; Van Dun et al., 1988; Stark and Beachy, 1989; Nejidat and Beachy, 1990), and absence or reduced accumulation virus level (Nelson et al., 1987; Cuozzo et al., 1988; Hemenway et al., 1988; Lawson et al., 1990) in the experimental transgenic plants developed against viruses in nine different groups (Beachy et al., 1990). In general, lack of visual symptoms is correlated to absence of virus level and an indication of transgenic plant resistant to virus infection. Such resistance obtained through plant transformation is not due to somaclonal variation and can be stably inherited. CP-MR was observed in transgenic plants against plant viruses in different virus group (Beachy et al., 1990).

CP-MR, a subset of pathogen-derived resistance, is well utilised in molecular breeding approach towards virus resistance before the discovery of RNA silencing mechanism (Lomonossoff, 1995; Prins et al., 2008) triggered by double-stranded (ds) RNA (Fire et al., 1998). The RNA

silencing revealed the small RNA world and potential function of the small RNA species as RNA interference had been proposed and demonstrated (Hamilton and Baulcombe, 1999; Elbashir et al., 2001a; Elbashir et al., 2001b). A dsRNA structure from viral sequence expressed in transgenic plants was able to induced RNA silencing and also granted virus immunity to the transgenic plants (Waterhouse et al., 1998). This method was used tested and use for generating crop resistance to plant viruses (Duan et al., 2012).

1.4.2 MicroRNAs and artificial microRNAs

1.4.2.1 MicroRNAs

In plants, there are several types of small RNA species and they are heterochromatic small interfering RNAs, long siRNAs, natural antisense transcript-derived siRNA, trans-acting siRNA and microRNAs (miRNAs) (Chapman and Carrington, 2007). The small RNAs are ubiquitous and versatile regulators of gene expression (Jones-Rhoades et al., 2006; Chen, 2010; Aalto and Pasquinelli, 2012), for instance involved in chromatic remodelling (Simons and Meyers, 2011; Olovnikov et al., 2012), development (Kidner and Martienseen, 2005; van Ex et al., 2011; Chen, 2012), genomic protect (Blumenstiel, 2011) and response to abiotic and biotic stress (Sunkar et al., 2007; Lu and Huang, 2008; Lu et al., 2008; Katiyar-Agarwal and Jin, 2010).

The synthesis and regulation of most miRNAs are well conversed in plant (Zhang et al., 2006a). MiRNAs are transcribed by RNA Polymerase II into 5'-capped and 3'-polyadenylated 100-1000 nt long primary transcripts (Jones-Rhoades et al., 2006). These primary transcripts will be processed into 60-70 nt precursor miRNAs with a stem loop structure by Dicer-like 1 (DCL1) (Bartel, 2004). The precursor miRNAs will then be exported out of nucleus and further processed into ~22 nt duplex by DCL1 (Xie et al., 2003; Bartel, 2004; Kidner and Martienseen, 2005). The duplex is loaded into RNA-inducing silencing complex (RISC) where only the mature miRNA strand, selected by Argonaute 1 (AGO1), remained to guide the RISC to silence the complementary target mRNAs (Jones-Rhoades et al., 2006). The silencing is achieved by mRNA cleavage and degradation though translation inhibition was reported (Helliwell and Waterhouse, 2005; Yu and Wang, 2010).

Plant miRNAs regulate transcription factors involved in biotic response such as infection by various types of pathogens like bacteria, fungi and viruses (Navarro et al., 2006; Mlotshwa et al., 2008). For virus infection, only bra-miR158 and bra-miR1885 was specifically upregulated when *Brassica rapa* was infected with *Turnip mosaic virus* (TuMV), as compared to other viruses like *Cucumber mosaic virus* (He et al., 2008).

Plant virus can evade the antiviral RNA silencing response by its encoded suppressors and cause developmental defects indirectly.

These RNA silencing suppressors can block AGO1 cleavage activity (Zhang et al., 2006b), or suppress gene silencing through interaction with supporessor of gene silencing 3 (Glick et al., 2008), or prevent the siRNAs duplex from entering the RISC (Vargason et al., 2003; Ye et al., 2003; Lakatos et al., 2006), thereby blocking the host RNA silencing mechanism (Wu et al., 2010; Saxena et al., 2011).

MiRNAs can be produced from viral origin using viral RdRp (Lu et al., 2008). These viral miRNAs can regulate viral and host gene expression (Sillivan and Ganem, 2005). The first reported viral miRNAs was encoded by the Epstein-Barr virus (Pfeffer et al., 2004) and followed by the discovery of other viral miRNAs (Cai et al., 2005; Sullivan et al., 2005).

The wide-ranging studies of plant and viral miRNAs offer a new direction towards silencing target gene effectively and bypass the difficulties encountered by other strategies (Sablok et al., 2011). Those strategies, such as virus-inducing gene silencing or overexpression of target gene, made use of the post transcriptional gene silencing pathway and relied on the production of siRNAs derived from dsRNAs (Sablok et al., 2011). The difficulties faced by these strategies include the need to develop a suitable viral vector and the risk of off-targets silencing or auto-silenced over time (Lu et al., 2003; Xu et al., 2006; Molnar et al., 2009).

1.4.2.2 Artificial microRNAs

The new direction was provided by artificial miRNAs (amiRNAs). The amiRNAs are designed based on the structure of an endogenous precursor miRNA and the endogenous miRNA region is replaced with desired miRNA sequence complementary to the target sequence. Thus, the biogenesis of modified precursor relies on the plant miRNA machinery. As plant miRNAs have high specificity, undesirable off-target effects can be avoided (Ossowski et al., 2008; Park et al., 2009). It was proposed that amiRNAs bring lesser biosafety or environmental issues for agricultural crops applications than other strategies (Liu and Chen, 2010). Furthermore, miRNAs are trans-acting and their silencing activity can be stably heritable to future generation (Ossowski et al., 2008; Zhao et al., 2008; Molnar et al., 2009). The small size of amiRNA allows the inclusion of multiple and unrelated amiRNAs within a single cassette to target specific alleles or unrelated genes (Niu et al., 2006; Schwab et al., 2010). The amiRNAs can be used to create transgenic plant resistant to one or more viruses simultaneously (Niu et al., 2006; Schwab et al., 2006). Niu, et al. (2006) modified a plant endogenous precursor miRNA, miR159, from *Arabidopsis thaliana* to express amiRNA targeting p69 of *Turnip yellow mosaic virus* (TYMV) and HC-Pro of TuMV. The resultant transgenic plants were able to resist against TYMV and TuMV challenge (Niu et al., 2006). It observed that the resistance

effect were more valuable than the ones obtained from short hairpin RNA (Qu et al., 2007). In another report, amiRNA were used to effectively silenced HC-Pro of *Potato virus Y* and TGBp1 (p25) of *Potato virus X* (Ai et al., 2010).

1.5 Gap of research and objectives of this project

Unique orchid flower traits and virus-free orchids are the long term goals of orchid culturists. Unique orchid flowers can be obtained through conventional sexual breeding and virus-free orchids can be achieved through meristem stem culture (Walkey, 1991; Hull, 2013). However, conventional breeding is slow and labour intensive and meristem stem culture to obtain virus-free orchids does not prevent subsequent virus infection.

The previous sections describe the plant pathology and the strategies to overcome the problems using existing plant transformation systems. From the reports on transgenic orchid studies, only two groups of researchers focused on the CymMV-resistant transgenic orchids using the virus coat protein (Liao et al., 2004; Chang et al., 2005). Due to the long vegetative growth of orchids, the results they had were based on T_0 transgenic orchids. And there are no follow-up reports on those CymMV-resistant T_0 transgenic orchids.

AmiRNAs provides an effective way to silence target gene with high specificity (Ossowski et al., 2008) and multiple targets gene simultaneously (Niu et al., 2006). The first objective is to design and create amiRNAs based on the sequence of CymMV and ORSV. The amiRNs from the viruses will be

contained within a single fragment so that both viral amiRNAs can be expressed in the plant cell simultaneously.

N. benthamiana is a common host for CymMV and ORSV. The second objective is to transform the viral amiRNAs transgene to generate *N. benthamiana* transgenic plants that are able to resist against CymMV and/or ORSV.

The use of genetic engineering provides a promising approach for orchid genetic improvement. No orchid that is either virus-resistant or unique flower traits is available commercially to meet the market demands. The third objective of this project is to try to generate transgenic *Dendrobium* Chao Praya Smile that can resist against CymMV and ORSV simultaneously. Orchids have a slower growth rate as compared to model plants such as *Arabidopsis thaliana* and *N. benthamiana*, and this slow growth rate poses a challenge with the limited time given.

Chapter 2 Materials and Methods

2.1 Plant materials and growth conditions

N. benthamiana and *Chenopodium amaranticolor* seeds, seedlings and developing plants were grown in potting mixture at 24^{o}C under a 16 h/8 h photoperiod regime of 35μmol· m^{-2}· sec^{-1} from daylight fluorescent lamps.

Seed germination, protocorms, putative transgenic plantlets of *Dendrobium* Chao Praya Smile were placed at 24^{o}C under a 16 h photoperiod of 35μmol· m^{-2}· sec^{-1} from daylight fluorescent lamp.

2.2 Development of *Dendrobium* Chao Praya Smile protocorms from seeds

Dendrobium Chao Praya Smile seed capsule of 3-month old was harvested. It was surface sterilized by 100% Clorox® bleach for 15 min before transferring to 100% ethanol for 15 min. Subsequently, the seed capsule was rinsed with sterile water thrice, each lasting for 5 min. Seeds were released from the capsule and sown onto germination medium (KC medium supplemented with 15% (v/v) coconut water, 3% (w/v) sucrose, 0.3% (w/v) activated charcoal, 0.3% (w/v) Phytagel®). Protocorms were expected to develop within two months.

2.3 Cloning of pG0229-preamiRNA-CymMV-ORSV and pG0229-antisense-CymMV-ORSV

2.3.1 Design of viral amiRNA

Design of viral amiRNA was achieved using Web MicroRNA Designer (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). The sequence of RNA-dependent RNA polymerase of CymMV and ORSV was input to the Designer field. From the result output, an amiRNA sequence of CymMV and ORSV was chosen.

2.3.2 Design of antisense CymMV-ORSV fragment

Based on the sequences of the amiRNA selected for the CymMV and ORSV fragments, an antisense fragment was designed each for CymMV and ORSV by selecting the sequences that were flanking the selected amiRNA sequence.

2.3.3 Polymerase chain reaction (PCR)

Each PCR reaction set-up for 25 µL in a 0.2 mL microcentrifuge tube was as followed: 1 × *Taq* enzyme buffer, 0.2 mM of dNTPs, 1 µM of appropriate primer, 1.25 U *Taq* polymerase and appropriate amount of DNA template. Amplification was performed in S1000™ Thermal Cycler (Bio-Rad Laboratories, Inc.) with a profile cycle of initial denaturation at 95^{o}C for 2 min, followed by 35 cycles of denaturation at 95^{o}C for 30 sec, annealing at temperature suitable to primers used for 30 sec, elongation at 72^{o}C for 30 sec, and a last step of extension at 72^{o}C for 5 min.

2.3.4 Purification of PCR products and DNA fragments from agarose gel

The PCR products were resolved by gel electrophoresis with 2% agarose gel which contained SafeView™ (Applied Biological Materials Inc.) for visualisation of DNA under Safe Imager™ 2.0 Blue-Light Transilluminator (Life Technologies). The desired fragment band was excised using a clean scalpel blade and its mass was determined by weighing scale. Nucleic acid extraction from the agarose gel was performed using Wizard® SV Gel and PCR Clean-up System (Promega). Membrane binding solution was added to the gel slice (10 µL: 10 mg) and the mixture was incubated at 60^{o}C until the gel sliced was completed dissolved. The mixture was transferred to the column provided, and washed with Membrane washing solution provided, and nucleic acid was eluted with sterile nuclease-free water. The concentration of the nucleic acid obtained was determined using NanoDrop 2000c spectrophotometer (Thermo Scientific).

2.3.5 PCR scheme to obtain amiRNA and antisense fragments

Primers to be used for cloning of amiRNA fragments were generated from the Web MicroRNA Designer (shown in Table 2.1). For obtaining the CymMV amiRNA fragments, three separate PCRs were performed first, each using pNW55 as template but with different pairs of primers to get three different DNA fragments. Primer C-5’ was paired with primer C-II to obtain fragment C-A; primer C-I was used with C-IV to produce fragments C-B; primer C-III was used together with primer C-3’ to

acquire fragment C-C. The three fragments were purified from the gel. Another round of PCR was performed using the three fragments as template along with primers C-5’ and C-3’ to result in a DNA fragment, C-amiRNA. As for obtaining ORSV amiRNA fragment, similar PCR steps were performed, except the primer pairs used were as follows: O-5’/O-II; O-I/O-IV; O-III/O-3’; O-5’/O-3’. Using both CymMV and ORSV’s amiRNA fragments as template with primer CO/C-5’/O-3’, a fused DNA fragment of CymMV-ORSV amiRNA was obtained and it was used for subsequent cloning.

Primers used for cloning of antisense fragments were listed in Table 2.2. After obtaining the antisense fragments for CymMV and ORSV, these fragments were used as template to obtain amtisene-CymMV-ORSV fragment and it was used for subsequent cloning.

Table 2.1 Primers used for cloning of amiRNA construct

Sequences in upper case indicate amiRNA and amiRNA* sequences introduced to the osa-MIR528 stemloop on backbone pNW55, and sequences in lower case indicate regions complementary to the backbone pNW55. Sequence italicised indicated the sequence of restriction enzyme.

Name of primer	**Sequence of primer (**5' to 3')
CymMV I miR-s	agTATAGCTCTACGTTTGGACAAcaggagattcagtttga
CymMV II miR-a	tgTTGTCCAAACGTAGAGCTATActgctgctgctacagcc
CymMV III miR*s	ctTTGTCGAAAGGTAGAGCTATAttcctgctgctaggctg
CymMV IV miR*a	aaTATAGCTCTACCTTTCGACAAagagaggcaaaagtgaa
ORSV I miR-s	agTTTTCGGGTTAAAAACCCCTTcaggagattcagtttga
ORSV II miR-a	tgAAGGGGTTTTTAACCCGAAAActgctgctgctacagcc
ORSV III miR*s	ctAAGGGCTTTATAACCCGAAAAttcctgctgctaggctg
ORSV IV miR*a	aaTTTTCGGGTTATAAAGCCCTTagagaggcaaaagtgaa
C5' *Eco*RI	tc*gaattc*cagcagcagccacagcaaa
O3' *Bam*HI	cgc*ggatcc*gctgctgatgctgatgccat
CO	ggctgctgctgcccgggggattccccggggctgctgatgc
35S	gacccttcctctatataaggaagttc
PGP2	ccttatcgggaaactactaacac

Table 2.2 Primers used for cloning of antisense construct

Sequence italicised indicated the sequence of restriction enzyme.

Name of primer	**Sequence of primer (**5’ to 3’)
CymMV 5' Antisense *Eco*RI	tc*gaattc*tcacctccacagggtc
CymMV 3' Antisense	ttctacttatcacccgaggggatc
ORSV 5' Antisense	gatttcgtgtacggtgttaatttcacc
ORSV 3' Antisense *Bam*HI	cgcggatccgttatcacttagatgcc
CymMV-ORSV Antisense link	tgaaattaacaccgtacacgaaatcttctacttatcacc
35S	gacccttcctctatataaggaagttc
PGP2	ccttatcgggaaactactaacac

2.3.6 Ligation of CymMV-ORSV amiRNA or antisense fragment into T-vector

Ligation reaction was performed according to the manufacturer's (Promega) instruction. The obtained CymMV-ORSV PCR fragment was ligated into T-vector, at a ratio of 1:1 in the presence of two units of T4 DNA ligase in 1 × ligation buffer. The ligation was carried out at 16^{o}C overnight in a 10 µL reaction volume.

2.3.7 Transformation of competent *Escherichia coli* with ligation mixture or plasmids

2.3.7.1 Preparation of chemically-competent *E. coli* DH5α

A single colony of DH5α was picked and grown in 2 mL Luria-Bertani (LB) and it was incubated at 37^{o}C shaker for 16 h. A volume of 1 mL of overnight culture was added to 100 mL fresh LB and left it in 37^{o}C with shaking until the OD_{600} reached between 0.4 and 0.6. The culture was placed on ice for 30 min. Subsequently, the cells were pelleted down at 6000 × *g* at 4^{o}C for 15 min. The cell pellet was resuspended with 10 mL of ice-cold 0.1 M $CaCl_2$ and left on ice for 30 min before centrifuged again. The cell pellet was the resuspended with 400 µL of 100% glycerol and 2 mL of ice-cold 0.1 M $CaCl_2$ before fractionated into 50 µL to a microfuge tube. The tubes containing the cell suspension were placed into liquid nitrogen (LN_2), followed by storage at -80^{o}C.

2.3.7.2 Transformation of DNA into chemically-competent *E. coli*

Competent cells were thawed on ice, the ligation mix was added and allowed the mixture to incubate on ice for 30 min. Heat-shock was performed at 42^{o}C for 90 sec followed by 2 min incubation on ice. To allow the cell to recover, 1 mL of LB was added and incubated the culture at 37^{o}C for 1 h without shaking. The cell was harvested by centrifugation at 6000 × *g* for 5 min following resuspension with 50 µL of LB. The cell mixture was spread onto LB plate containing 50 µg/mL of ampicillin for selection. The plate was placed in 37^{o}C incubator overnight to allow colony formation.

2.3.8 Plasmid isolation

Single bacterial clones that grew on LB plate containing antibiotic was inoculated into 2 mL LB with 50 µg/mL of ampicillin. The cultures were left in 37^{o}C shaker overnight. Plasmids extraction was performed according to the instruction provided by Axyprep Plasmid Miniprep Kit (Axygen Biosciences). Bacterial cells were collected by centrifugation at 14000 × *g* for 1 min at room temperature. Buffer S1 containing RNase (250 µL) was used to resuspend the cell pellet. Cell lysis was achieved after the addition of Buffer S2 (250 µL) and inverted the tube gently for eight times. Buffer S3 was added (350 µL) and mixed by inverting the tube gently for eight times before centrifugation for 14000 × *g* for 10 min at room temperature. The cell lysate was then transferred to the Miniprep column attached to a collection tube (provided by the kit) and centrifuged at 14000 × *g* for 30 sec at room temperature. Subsequently, Buffer W1

(500 µL) and Buffer W2 (700 µL) was used to wash the column by centrifugation at 14000 × *g* for 1 min at room temperature. The column was transferred to a microfuge tube and sterile water (30 µL) was added to the centre of the column and allowed the column to stand for 5 min. DNA was eluted from the column by centrifugation at 14000 × *g* for 1 min at room temperature. The concentration of the eluted plasmid was determined by NanoDrop 2000c spectrophotometer.

2.3.9 Sequence validation

Nucleotide sequences of the constructs were verified by sequencing before downstream usages. Sequencing was carried out by amplification using Bigdye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on S1000™ Thermal Cycler (Bio-Rad Laboratories, Inc.). A 10µL reaction setup is as follows: 1 µL of BigDye (ABI Prism^{TM} Dye), 2 µL of 5 × sequencing buffer, 0.2 µM primer, appropriate amount of plasmid as template (10 ng per 1 kb template) and top-up with sterile water. The reaction mixture was subjected to PCR at initial denaturation at 96^{o}C for 1 min, 30 cycles of denaturation at 96^{o}C for 10 sec, annealing at 50^{o}C for 5 sec and extension at 60^{o}C for 1 min, with final extension at 60^{o}C for 5 min. To the PCR products, 1 µL of 3 M NaOAc and 25 µL of 95% ethanol was added and the overall mixture was vortexed and left to stand for 5 min at room temperature. The mixture was spun at 14000 × *g* for 15 min and the supernatant was removed. The pellet was washed with 500 µL of 75% ethanol and centrifuged at 14000 × *g* for 15 min. The DNA pellet was subjected to vacuum drying. The dried pellet was sent to

the Sequencing Laboratory, Department of Biological Sciences, National University of Singapore for sequencing determination by ABI Prism 3100 automated sequencer. Sequences of the desired target genes were verified to contain no undesired mutations. The plasmid containing the correct sequence of target genes was kept for further experiments.

2.3.10 Restriction digestion of preamiRNA-CymMV-ORSV and antisense CymMV-ORSV from T-vector

Based on the sequencing results, plasmids that did not have mutations were used for restriction enzyme digestion. *Eco*RI and *Bam*HI (New England Biolabs) were used to digest the plasmids. Empty binary vector, pG0229 was also used for restriction enzyme digestion using the same restriction enzymes pair. Digestion was carried out in 100 µL containing 1 × NEB Buffer 3, 0.5 U *Eco*RI, 0.5 U *Bam*HI and 10 µg of plasmid. After digestion at 37^{o}C for 1 h, the digested products were purified from agarose gel. Ligation of preamiRNA-CymMV-ORSV or antisense CymMV-ORSV into pG0229 was performed. The ligation mix was transformed into DH5α competent cells. Plasmids were isolated from single colony that grew on LB containing 50 µg/mL of kanamycin.

2.3.11 Transformation of *A. tumefaciens* GV3101 strain

2.3.11.1 Preparation of competent Agrobacteria cells

A single colony was picked and cultured in 2 mL of LB containing 10 µg/mL of tetracycline, 25 µg/mL of gentamycin and 50 µg/mL of rifampicin and incubated in 28^{o}C shaker overnight. The

Agrobacteria culture was transferred to 100 mL of LB supplemented with the antibiotics and incubated in the shaker till OD_{600} reached 0.6. Next, the cells were kept on ice for 20 min before centrifuged at 6000 × *g* for 10 min at 4°C. The Agrobacteria cell pellet was resuspended with 8 mL of ice-cold sterile water and left it on ice for 10 min. Agrobacteria cell suspension was fractioned into 100 µL to microfuge tube and frozen with LN_2 prior to storage in -80°C freezer till further use.

2.3.11.2 Transformation of plasmid DNA into GV3101

Ice-thawed Agrobacteria competent cells was introduced with pG0229-preamiRNA-CymMV-ORSV or pG0229-antisense-CymMV-ORSV (1 µg each) and placed in LN_2 for 2 min before placing it in 37°C incubator for 5 min. A volume of 1 mL of LB was added and incubated at 28°C for 4 hrs. Supernatant (850 µL) was removed after centrifugation at 6000 × *g* for 2 min. The bacterial cells were spread onto LB plate containing 50 µg/mL of kanamycin, 50 µg/mL of rifampicin, 10 µg/mL of tetracycline and 25 µg/mL of gentamycin and incubated at 28°C for 48 h to allow colony formation.

2.4 *Agrobacterium*-infiltration

A single colony of Agrobacteria was inoculated into 2 mL LB containing 50 µg/mL of kanamycin, 50 µg/mL of rifampicin, 10 µg/mL of tetracycline and 25 µg/mL of gentamycin and incubated overnight with shaking at 28°C. The

next day, 20 µL of the culture was transferred into 25 mL of LB containing the same antibiotics overnight with shaking at 28^{o}C until the OD_{600} reached 1.0, determined by UV/Vis Spectrophotometer (Beckman, USA). The Agrobacteria cells were harvested by centrifugation at 6000 × *g* for 10 min at room temperature and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 10 µM acetosyringone) for 4 h. The infiltration mixture was agroinfiltrated into the underside of *N. benthamiana* leaves. The plants were placed in dark overnight before returning to normal growth conditions, as mentioned in Section 2.1.

2.5 *Agrobacterium*-mediated transformation

2.5.1 Preparation of Agrobacteria culture

Agrobacterium cells harbouring construct of interest were inoculated into 2 mL LB broth containing containing 50 µg/mL of kanamycin, 50 µg/mL of rifampicin, 10 µg/mL of tetracycline and 25 µg/mL of gentamycin and incubated at 28^{o}C overnight before taking 50 µL of culture out the next day and inoculated into 20 mL of LB broth containing the mentioned antibiotics to an OD_{600} of 0.6.

2.5.2 Agrotransformation of *N. benthamiana* leaf explant

N. benthamiana leaves were surface sterilized with 0.8% Clorox® bleach for 10 min followed by rinsing with sterile water thrice, each lasting for 5 min. The explants of 1 cm^2 were cut out and co-cultivated with Agrobacteria culture (OD_{600} of 0.6), diluted 4 × with ½ MS medium for 30 min. Subsequently, the explants exposed to Agrobacteria culture were

blotted dried with sterile filter paper before placing them on ½ MS plates containing 100 µM acetosyringone. The culture plates were placed in dark for three days. After three days, the explants were washed with water containing 100 mg/L carbencillin thrice, each lasting for 5 min. The explants were rinsed with sterile water thrice, each lasting for 5 min before blotting dry with sterile filter paper and placed them on callus induction medium (½ MS plate with 1.5 mg/L BA, 0.1 mg/L NAA, 100 mg/L carbenicillin and 20 mg/L PPT). The plant tissue cultures were placed at 24^{o}C under a 16 h photoperiod of 35µmol· m^{-2}· sec^{-1} from daylight fluorescent lamp.

Leaf explants were subcultured every two weeks to ensure enough nutrients were provided. Putative transgenic calli that grew from the explants on the callus induction medium were distributed into smaller cluster and placed on shoot induction medium (½ MS plate with 1.5 mg/L BA, 100 mg/L carbenicillin, 20 mg/L PPT). Once shoot growth was observed, it was transfer to ½ MS plate for root formation.

When roots were observed from putative transgenic *N. benthamiana* shoots, the plantlets were potted to soil, with a layer of plastic wrap overlay to maintain high humidity for a week. After a week, the putative transgenic *N. benthamiana* plantlets were left to grow, and transgenic seeds were harvested.

2.5.3 Agrotransformation of *Dendrobium* Chao Praya Smile protocorms and screening of transformants

Agrobacteria culture (5 mL) was added to 45 mL of protocorms-containing KC liquid medium with 10 mM MES, 10 µM acetosyringone. *Dendrobium* Chao Praya Smile protocorms were incubated with Agrobacteria cell culture for 3 h at 24^{o}C under light with constant shaking. After incubation period, the protocorms were blotted dried and were placed on KC plate supplemented with 1 mg/L BA and 100 µM AS, for three days of co-cultivation. The co-cultivation period is to promote T-DNA transfer and in turn increase the transformation efficiency.

After three days, the protocorms were rinsed with sterile water containing 100 mg/L carbenicillin, each lasting 5 min, before placing the protocorms on KC plate supplemented with 1 mg/L BA and 100 mg/L carbenicillin for three days. Another round of washing was performed before placing the protocorms on KC plate supplemented with 1 mg/L BA, 100 mg/L carbenicillin and 2 µM MSO. Subculture of putative transformants was carried out every three weeks. During each subculture, necrotic tissues that did not manage to grow on the selection medium were removed.

2.5.4 Agrotransformation of *Dendrobium* Chao Praya Smile pollinia

Agrobacteria culture was diluted 4 × with KC liquid medium supplemented with 10 mM MES and 10 µM acetosyringone. *Dendrobium* Chao Praya Smile pollinia were soaked in the diluted Agrobacteria culture for 30 min before pollination was performed. Protocorms obtained (according to Section 2.2) was transferred to KC plate containing 1 mg/mL

BA and 2 µM MSO. Subculture of putative transformants was carried out every three weeks. During each subculture, necrotic tissues that did not manage to grow on the selection medium were removed.

2.5.5 Particle bombardment of *Dendrobium* Chao Praya Smile pollinia

Pollinia used were slightly squashed and placed on wet filter paper. Preparation of the plasmid DNA-coated gold particles was according to Seashell Technology Plasmid DNA Binding Particle Formulation Protocol (Seashell Technology LLC). Bombardment was carried out twice using Model PDS-1000/He Biolistic® particle Delivery System (Bio-Rad Laboratories, Inc.) with the following parameters: the bombardment chamber was evacuated at a pressure of 27 inches Hg; the distance of 6 cm from the stopping screen to the surface of the samples; the helium gas pressure ranges from 650 to 1550 psi; an amount of 3 µg of plasmid DNA was attached to 1 mg of gold particles of 1 nm in diameter. Another set of bombardment was also carried out twice, with only the parameter of the distance from the stopping screen to the surface of the samples changed from 6 cm to 9 cm. A total of seven *Dendrobium* Chao Praya Smile orchid flowers' pollinia were used for each bombardment. The bombarded pollinia were used for pollination. Seed capsules formed were harvested and seeds were released to the germination medium containing 0.2 µM MSO. Putative transformants were then subcultured every three weeks to KC plate containing 1 mg/mL BA and 2 µM MSO. The whole process was repeated once.

2.6 Analysis of DNA

2.6.1 Genomic DNA extraction

Leaves from putative transgenic *N. benthamiana* plants or *Dendrobium* Chao Praya Smile were ground with liquid nitrogen. The ground powder was transferred to a pre-chilled 2 mL microfuge tube and 1 mL (per 0.2 g of fresh leaves) of CTAB extraction buffer (2% CTAB, 0.1 M Tris-Cl, pH 7.0, 1.4 M NaCl, 20 mM EDTA, 0.3% sodium lauryl sarcosine) was added. The homogenised mixture was incubated at 65^{o}C for 40 min, with gentle inversion every 10 min. The mixture added with 700 µL of chloroform followed by vigorously shaking. The emulsion was centrifuged at 10000 × *g* for 10 min at room temperature. The aqueous phase was carefully pipetted out and transfer to an empty microfuge tube. One-tenth volume of 10% (w/v) CTAB was added to the aqueous phase and incubated at 65^{o}C for 20 min before equal volume of chloroform was added. The mixture was spun at 10000 × *g* for 10 min at room temperature. The top aqueous phase was transferred to a new microfuge tube. One-tenth volume (V) of 3 M NaOAc, pH 5.2, was added to the aqueous phase followed by 2.5V of 95% ethanol. The mixture was inverted a few times and kept it at 4^{o}C overnight. The next day, the mixture was centrifuged at 10000 × g for 20 min at room temperature. The supernatant was decanted and 70% ethanol was used to wash the pellet followed by centrifugation at 10000 × *g* for 10 min at room temperature. The DNA pellet was allowed to air-dry before dissolving it with sterile water.

2.6.2 PCR analysis

Genomic DNA extracted from putative transgenic *N. benthamiana* or *Dendrobium* Chao Praya Smile was used as template for detecting presence of transgene. PCR set-up was in according to Section 2.3.2 and the PCR products were resolved by gel electrophoresis with 2% agarose gel which contained SafeView™ (Applied Biological Materials Inc.) for visualisation of DNA under Safe Imager™ 2.0 Blue-Light Transilluminator (Life Technologies).

2.6.3 Southern blot

2.6.3.1 DIG-labelled probes for Southern blot

A DIG-labelled probe complementary to the sequence of the transgene was synthesized using PCR DIG Probe Synthesis Kit (Roche) according to the manufacturer's instructions.

2.6.3.2 Restriction enzyme digestion

The genomic DNA extracted (20 µg) was subjected to *Eco*RI digestion over the night. The digested genomic DNA was recovered by ethanol precipitation. In brief, one-tenth volume of 3 M NaOAc, pH 5.2, along with 2.5 V of 95% ethanol was added to the restriction digestion reaction mixture. This new mixture was gently inverted a few times before placing it in -20^{o}C freezer overnight. In the following day, the mixture was centrifuged at 10000 × g for 10 min at room temperature. The supernatant was decanted and 70% ethanol was used to wash the pellet followed by centrifugation at 10000 × g

for 10 min at room temperature. The DNA pellet was allowed to air-dry before dissolving it with sterile water.

2.6.3.3 Gel electrophoresis and treatment of the gel for capillary transfer

*Eco*RI-digested DNA samples were subjected to gel electrophoresis was conducted at 20 V until the visible dye went two-third of the gel length. After the end of gel electrophoresis, the excess area at the sides of the gel was trimmed off. The trimmed gel was treated with denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 15 min at room temperature twice. The gel was rinsed with sterile water before submerged in neutralization buffer (0.1 M Tris-Cl, pH 7.5) for 15 min at room temperature twice. Following which, the gel was equilibrated with 20 × SSC (3 M NaCl, 0.3 M trisodium citrate) for 20 min.

2.6.3.4 Capillary transfer setup

The digested genomic DNA in the gel was transfer to nylon positively charged membrane (Roche) via capillary transfer method. In brief, the treated gel was placed on top of a soaked filter paper lying on a bridge with a 20 × SSC reservoir underneath. The dry membrane was placed carefully on top of the gel without any bubbles in between. A dry piece of filter paper was placed on top of the membrane followed by a stack of paper towels with 300 g of weight on top. This assembly was left overnight to allow transfer of digested genomic DNA to membrane.

2.6.3.5 Hybridisation of DIG-labelled probe and detection

The DNA was UV-crosslinked to the membrane in the presence of UV (120 mJ burst over 30 sec). After which, the membrane was put in prehybridisation buffer for 1 h with gentle shaking. Hybridisation buffer, containing the DIG-labelled probe prepared in Section 2.6.3.1, replaced the prehybridisation buffer and incubated at 42^{o}C overnight. Low stringency buffers (2 × SSC, 0.1% SDS) were used to wash the membrane at room temperature for 15 min twice. The membrane was then washed with preheated high stringency buffer (0.1 × SSC, 0.1% SDS) at 65^{o}C for 15 min twice. The membrane was briefly rinsed with washing solution [Maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), 0.3% Tween-20]. Blocking solution (10% (w/v) blocking reagent from Roche dissolved in Maleic acid buffer) was poured onto the membrane and left it with gentle shaking for 1 h. Anti-DIG antibody (1:5000 dilution, Roche) was added to the blocking solution and the membrane was incubated for 1 h. Washing solution was used to wash the membrane for 15 min at room temperature twice. Development buffer (0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl) was used to equilibrate the membrane. CSPD (Roche) was dripped onto the membrane and left it in 37^{o}C incubator for 30 min. The membrane was exposed to chemilluminescent film for a week before developing the film.

2.7 Analysis of amiRNA-CymMV-ORSV transgene expression in transgenic *N. benthamiana* plants

2.7.1 Total RNA extraction using TRIzol®

Leaf tissues were homogenized with 1 mL of TRIzol® (per 100 mg of fresh leaf tissues). The homogenised samples were placed at room temperature for 5 min before the addition of 0.2 mL of chloroform (per 1 mL of TRIzol®). The mixture was shook vigorously for 15 sec and incubated at room temperature for 3 min before centrifuged at 16000 × *g* for 15 min at 4°C. The aqueous phase was pipetted carefully to a new microfuge tube containing 0.5 mL of isopropanol, for every 1 mL of TRIzol® used. The mixture was incubated for 10 min at room temperature before centrifugation at 16000 × *g* for 10 min at 4°C. The total RNA pellet was washed with 1 mL of 75% ethanol, for every 1 mL of TRIzol® used. The washed total RNA pellet was air-dried before dissolving with 20 µL of sterile water. The concentration of the dissolved total RNA was determined by NanoDrop 2000c spectrophotometer.

2.7.2 Detection of small RNA by Northern blot

An equal volume of RNA Gel Loading Bufer II (Ambion®) was added to 20 µg of total RNA. The mixture was then denatured at 100°C for 5 min and chilled on ice. The denatured samples were loading into a denaturing 8 M urea- 15% polyacryladmide gel and electrophroresed at a constant 200 V. When the bromophenol blue dye had migrated two-third of the gel, electrophoresis was stopped. The total RNA was transferred to a positive-

charged nylon membrane (Roche) by electroblotting at constant 200 mA for 2 h. The total RNA was UV-crosslinked onto the damp membrane (with 120 mJ burst over 30 sec). The membrane was placed in prehybridisation buffer at 42^{o}C for at least 1 h. The membrane was then next incubated in hybridisation buffer (containing appropriate 3'- end DIG labelled probe) at 42^{o}C overnight. Subsequently the membrane was washed with wash solution (6 × SSC, 0.2% SDS) thrice at 42^{o}C for 10 min. Blocking solution (Roche) was added to the membrane and incubated for at least 1 h at room temperature. Anti-DIG antibody was added to the blocking solution and incubated at room temperature for 1 h. Membrane was washed with Washing solution thrice at room temperature for 10 min before. The membrane was equilibrated with development buffer before the addition of CSPD (Roche) and incubated at 37^{o}C for 30 min. The membrane was exposed to chemilluminescent film for a day before developing the film.

2.8 Infectivity analysis CymMV and ORSV *in vitro* transcripts

CymMV *in vitro* transcripts and ORSV *in vitro* transcripts were synthesised using mMESSAGE mMACHINE® T7 Transcription Kit (Ambion®) according to the manufacturer's instructions. The concentration of the viral *in vitro* transcripts obtained was quantified using NanoDrop 2000c spectrophotometer. An amount of 500 ng of CymMV *in vitro* transcripts and ORSV *in vitro* transcripts were inoculated onto the leaves of *C. amaranticolor* and *N. benthamiana*. The virus inoculated plants were observed for any local lesions or viral symptoms and systemic infection.

2.9 Inoculation of viral *in vitro* transcripts to transgenic *N. benthamiana* plants

An amount of 500 ng of CymMV *in vitro* transcripts or ORSV *in vitro* transcripts, singly or simultaneously, were inoculated to transgenic *N. benthamiana* plants when they had six fully expanded leaves. The virus challenge was carried out thrice with three biological repeats for each treatment. Wild-type *N. benthamiana* was inoculated with PBS solution (1.37 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) as a negative control.

2.10 Analysis of total RNA and total proteins of the virus-inoculated transgenic *N. benthamiana* plants 21 days post inoculation

2.10.1 cDNA synthesis

After 21 days post inoculation, half of the first fully expanded leaf was harvested. Extraction of total RNA was carried out according to Section 2.7.1. Total RNA (~2 µg) was used for reverse transcription to generate complementary DNA (cDNA) using SuperScript™ III Reverse Transcriptase (RT) (Invitrogen, USA) according to the manufacturer's instructions. In brief, the RT reaction, containing 2 pmol of gene specific primers, 1 µL of 10 mM dNTPs mix, 2 µg of total RNA and water, was heated at 65°C for 5 min. subsequently, the RT reaction was placed on ice for 2 min before the addition of 4 µL of 5x First Strand Buffer, 1 µL of 0.1 M dithiothreitol (DTT), 1 µL of Superscript™ III RT. The entire RT

reaction was incubated at 55^{o}C for 60 min. The deactivation of Superscript™ III RT was achieved by heating t 70^{o}C for 5 min.

2.10.2 RT-PCR

The cDNA products were used as template for RT-PCR to quantify the gene expression levels. The PCR reaction set-up and profile was similar to Section 2.3.2 except the template used was cDNA products. Actin gene of *N. benthamiana* was used as internal control.

2.11 Analysis of presence of virus coat protein in virus-challenged transgenic *N. benthamiana* plants 21 days post inoculation

2.11.1 Total proteins extraction

Total proteins were extracted using the half of the first fully expanded leaf with one volume of protein extraction buffer (0.22 M Tris-HCL, pH 7.4, 250 mM sucrose, 50 mM KCl, 1 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride and 10 mM β-mercaptoethanol].

2.11.2 Determination of protein concentration using Bradford Protein Assay

The Bio-Rad Protein Assay Dye Reagent Concentrate (5 ×) was diluted with sterile water prior to use. A series of different bovine serum albumin amount (0, 2, 4, 6, 8, 10 µg) was used for standard curve. The total proteins extracted (2 µL) was added to 1 mL of diluted assay dye and

mixed well and incubated at room temperature or 5 min. The absorbance readings of the standards, and protein samples were mueasured at 595 nm.

2.11.3 SDS-polyacrylamide electrophoresis (PAGE)

A vertical SDS-PAGE gel gel apparatus from Bio-Rad Laboratories, Inc. was assembled based on the manufacturer's instructions and a 12% resolving gel overlaid with 3% stacking gel with 10 wells was prepared. An amount of 10 µg of total proteins extracted were denatured at 100^{o}C water bath in the presence of SDS loading dye [0.25% bromophenol blue, 0.5 M DTT, 50% glycerol, 10% (w/v) SDS] before incubation on ice for 5 min. It was then centrifuged at 12000 × *g* for 5 min at 4^{o}C. The denatured proteins samples were loaded into the well and electrophoresis was conducted at a constant voltage of 80V for 150 min in the presence of electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) The protein gels were either stained with Coomassie Brillant Blue staining solution [50% ethanol, 0.025% (w/v) Coomassie Blue R-250, 0.75% (v/v) glacial acetic acid] or used for Western blot.

2.11.4 Western blot

The protein from the protein gel after electrophoresis was transferred to nitrocellulose membrane using a wet electroblotting system (Bio-Rad Laboratories, Inc.). The assembly of the wet electroblotting system was according to the manufacturer's instruction. The protein transfer was conducted at a constant current of 200 mA for 1 h under chilled condition in the presence of transfer buffer [20% (v/v) methanol, 50 mM Tris, 380 mM glycine, 0.1% SDS]. The nitrocellulose membrane was treated with 5%

non-fat milk in 1 × PBS solution for 30 min with shaking at room temperature. The nitrocellulose was next incubated in the presence of appropriate primary rabbit antibody (anti-CymMV CP or anti-ORSV CP) for 1 h with shaking at room temperature. Subsequently, the membrane was washed thrice with 1 × PBS solution, 5 min at room temperature each time. Anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) was added to the membrane in presence of 1 × PBS buffer and incubated for 1 h with shaking at room temperature. The membrane was washed thrice before equilibrated with developing buffer. Colour development reaction was carried out by addition of NBT/BCIP and the membrane was left in the dark. The reaction was stopped when bands were distinct by rinsing the membrane with sterile water.

Chapter 3 Results

3.1 Dual viral amiRNAs and antisense fragments incorporated into pGreen vector

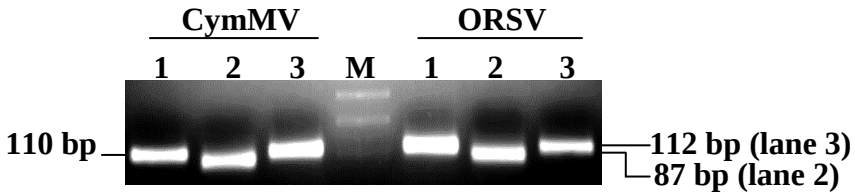
The RdRp of CymMV and ORSV were used as the target genes to create viral amiRNAs. The RdRp sequences of CymMV and ORSV were input into the Web MicroRNA Designer tool and the tool generated a list of potential amiRNAs. Considering no potential off-targets and low thermostability, the CymMV amiRNA sequence chosen was "TATAGCTCTACGTTTGGACAA" and the ORSV amiRNA sequence selected was "TTTTCGGGTTAAAAACCCCTT". A list of primers was generated based on the viral amiRNA chosen (Table 2.1) to be used for creating the construct pG0229-preamiRNA-CymMV-ORSV.

Firstly, site-mutagenesis PCR was performed to replace the original microRNA sequence in the pNW55 template and three different PCR fragments for CymMV amiRNA and for ORSV amiRNA were obtained (Figure 3.1A). Next, overlapping PCR was carried out by using the three fragments obtained for CymMV amiRNA as template to produce a fragment of 261 bp in length (Figure 3.1B). This process was repeated for ORSV amiRNA (Figure 3.1A-B). Subsequently, the CymMV amiRNA fragment and ORSV amiRNA fragment were used as template to link the two fragments together to generate a CymMV-ORSV amiRNA fragment of 522 bp in length (Figure 3.1C). After ensuring the sequence of the CymMV-ORSV amiRNA fragment was correct, the fragment was eventually inserted into a pG0229

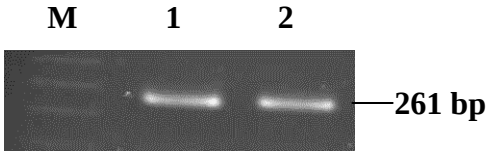
vector to become pG0229-preamiRNA-CymMV-ORSV construct (Figure 3.1D, E). The construct was transformed into *A. tumefaciens* GV3101 strain and the Agrobacteria cells were used for agroinfiltration and *Agrobacterium*-mediated transformation.

After obtaining the two antisense fragments for CymMV and ORSV (Figure 3.2A), overlapping PCR was carried out to link the two fragments together along with the introduction of restriction enzyme site, *Eco*RI to the 5' end of CymMV antisense fragment and *Bam*HI to the 3' end of antisense fragment (Figure 3.2B). Once the CymMV-ORSV antisense fragment was obtained from PCR, it was subjected to restriction enzyme digestion before ligated to pG0229 vector. The ligation products were transformed into competent *E. coli* strain DH5α via heat-shock approach. Putative colonies that grew on LB plates with 50mg/L kanamycin were picked and plasmid extraction was performed. Sequencing was carried out to verify no mutation was introduced during PCR. The construct, pG0229-CymMV-ORSV antisense, was introduced into *A. tumefaciens* strain GV3101 cells via heat-shock approach. Plasmid extraction was performed from putative clones and PCR was carried out, using 35S and PGP2 primers, to ensure pG0229-CymMV-ORSV antisense was present in the Agrobacteria cells (Figure 3.2C, D).

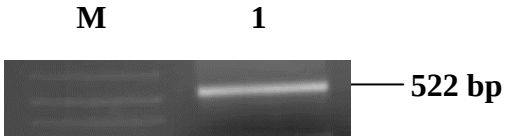
A


CymMV
ORSV
1 2 3 M 1 2 3
110 bp
112 bp (lane 3)
87 bp (lane 2)

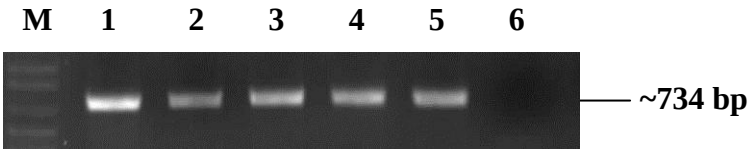
B


M 1 2
261 bp

C


M 1
522 bp

D


M 1 2 3 4 5 6
~734 bp

E

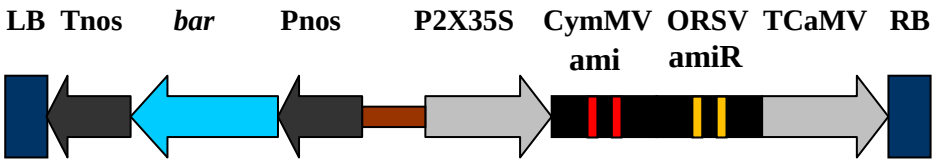

LB
Tnos
bar
Pnos
P2X35S
CymMV ami
ORSV amiR
TCaMV
RB

Figure 3.1 Construction of pG0229-preamiRNA-CymMV and ORSV

(A) Using pNW55 as plasmid template, site directed mutagenesis was performed to include amiRNA and amiRNA* sequences of CymMV and ORSV to obtain products A (lane 1), B (lane 2), and C (lane 3), respectively. Lane M represents 1 kb DNA ladder.

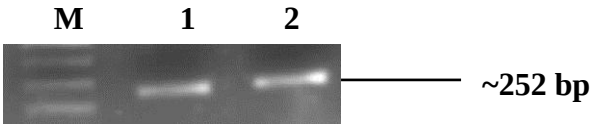
(B) After obtained products A, B, C for CymMV and ORSV respectively, the products were pooled individually to obtain amiRNA precursor for CymMV (lane 1) and ORSV (lane 2) respectively. Lane M represents 1 kb DNA ladder.

(C) AmiRNA precursors of CymMV and ORSV were used as template to generate amiRNA precursor of CymMV-ORSV (lane 1). Lane M represents 1 kb DNA ladder.

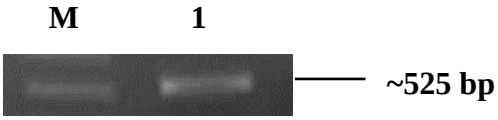
(D) AmiRNA precursor of CymMV-ORSV was inserted into vector pG0229 and resulting construct, pG0229-pre-amiRNA-CymMV-ORSV, was transformed into *Agrobacterium tumefaciens* GV3101 strain. Plasmids were extracted from several *A. tumefaciens* colonies that grew on selection medium and PCR was performed to verify insert is present in the vector (lane 1 to 5). Lane 6 is a negative control using pG0229 empty vector. Lane M represents 1 kb DNA ladder.

(E) Schematic diagram of the T-DNA region of the construct, pG0229-pre-amiRNA-CymMV-ORSV. **LB**, left border; **RB,** right border; **Pnos**, nopaline synthase promoter sequence; **Tnos**, nopaline synthase terminator sequence; ***bar***, bialaphos resistance gene; **P2X35S**, double Cauliflower mosaic virus 35S promoter sequence; **TCaMV**, Cauliflower mosaic virus terminator sequence; **CymMV amiRNA**, amiRNA sequence of the RdRp of CymMV (in red) and it is followed by **ORSV amiRNA**, amiRNA sequence of the RdRp of ORSV (in yellow).

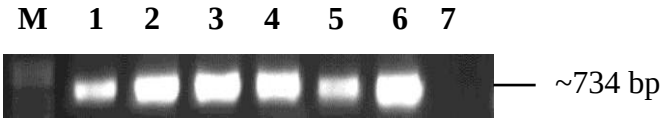
A


M
1
2
~252 bp

B


M
1
~525 bp

C


M 1 2 3 4 5 6 7
~734 bp

D

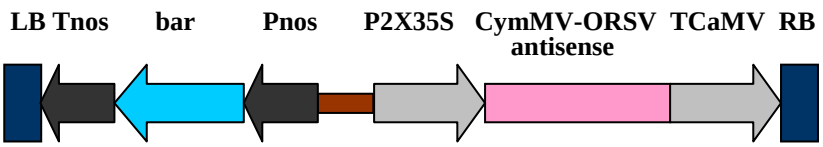

LB Tnos
bar
Pnos
P2X35S
CymMV-ORSV antisense
TCaMV
RB

Figure 3.2 Generation of antisense segments of CymMV and ORSV and verification of insert within vector pG0229.

(A) Using infectious clones of CymMV and ORSV, the antisense region of RdRp of CymMV (lane 1) and ORSV (lane 2), respectively, was successfully amplified. Lane M represents 1 kb DNA ladder.

(B) The amplified antisense segments of CymMV and ORSV were used as templates to obtained CymMV-ORSV antisense fragment. Lane M represents 1 kb DNA ladder.

(C) CymMV-ORSV antisense fragment was inserted into vector pG0229 to obtain resulting construct, pG0229-CymMV-ORSV antisense, and was transformed into *Agrobacterium tumefaciens* GV3101 strain. Plasmids were extracted from several *A. tumefaciens* colonies that grew on selection medium and PCR was performed to verify insert is present in the vector (lanes 1 to 6). Lane 7 is a negative control using pG0229 empty vector. Lane M represents 1 kb DNA ladder.

(D) Schematic diagram of the T-DNA region of the construct, pG0229-pre-amiRNA-CymMV-ORSV.**LB**, left border; **RB,** right border; **Pnos**, nopaline synthase promoter sequence; **Tnos**, nopaline synthase terminator sequence; ***bar***, bialaphos resistance gene; **P2x35S**, double Cauliflower mosaic virus 35S promoter sequence; **TCaMV**, Cauliflower mosaic virus terminator sequence; **CymMV-ORSV antisense**, antisense sequence of RdRp of CymMV linked to antisense sequence of RdRp of ORSV.

3.2 Expression of dual viral amiRNAs in agroinfiltrated samples

Agroinfiltration was performed to determine if the viral amiRNAs were able to express in the target plant cell. Agroinfiltration was carried out on three different wild-type *N. benthamiana* plants. Three days post infiltration, total RNA were extracted from infiltrated *N. benthamiana* leaves and RT-PCR was performed. PCR products were observed from samples that were agroinfiltrated with Agrobacteria cells harbouring pG0229-preamiRNA-CymMV-ORSV and no band was observed from sample agroinfiltrated with Agrobacteria cells harbouring pG0229 empty vector (Figure 3.3). This showed that transgene, amiRNA-CymMV-ORSV, was able to express in target plant cells after agroinfiltration.

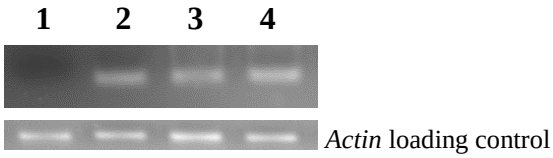


Figure 3.3 RT-PCR products of *Nicotiana benthamiana* leaves agroinfiltrated with pG0229-preamiRNA-CymMV-ORSV.

Three different wild-type *N. benthamiana* plants were agroinfiltrated with pG0229-preamiRNA-CymMV-ORSV and transgene was detected (Lane 2-4). No transgene was detected when wild-type *N. benthamiana* was agroinfiltrated with pG0229 empty vector (Lane 1).

Lane 1, Leaf agroinfiltrated with pG0229 empty vector; Lanes 2-4, different leaf samples agroinfiltrated with pG0229-preamiRNA-CymMV-ORSV. Actin gene was used as loading control.

3.3 Putative T_0 transgenic *N. benthamiana* harbouring pG0229-preamiRNA-CymMV-ORSV or pG0229-antisense-CymMV-ORSV

After determined the viral amiRNAs were able to express in the target plant cell through agroinfiltration, the next step was to generate transgenic *N. benthamiana* to test the CymMV and/or ORSV resistance capability.

Leaves from *N. benthamiana* were surface-sterilised and cut into 1 cm^2 in size and used for *Agrobacterium*-mediated transformation. After the leaf explants were soaked in Agrobacteria culture for 30 min, they were blotted dried and placed on ½ MS plate supplemented with 1 mg/L BA, 0.1 mg/L NAA and 20 mg/L phosphinotricin to induce callus formation and to screen for putative transgenic transformants (Figure 3.4A). After a month of subculturing, putative transgenic amiRNA-CymMV-ORSV and antisense-CymMV-ORSV calli were observed (Figure 3.4B, C). Some of the putative transgenic amiRNA-CymMV-ORSV and antisense-CymMV-ORSV calli differentiated into shoot (Figure 3.4D, E) and they were transferred to fresh ½ MS plate for root formation. When roots were observed to be growing from the putative transgenic shoots after a month on ½ MS medium (Figure 3.4F), the small putative transgenic amiRNA-CymMV-ORSV and antisense-CymMV-ORSV *N. benthamiana* plantlets were potted into soil for proper growth and seed setting (Figure 3.4G, H). These putative transgenic plants were considered as T_0 transgenic generation.

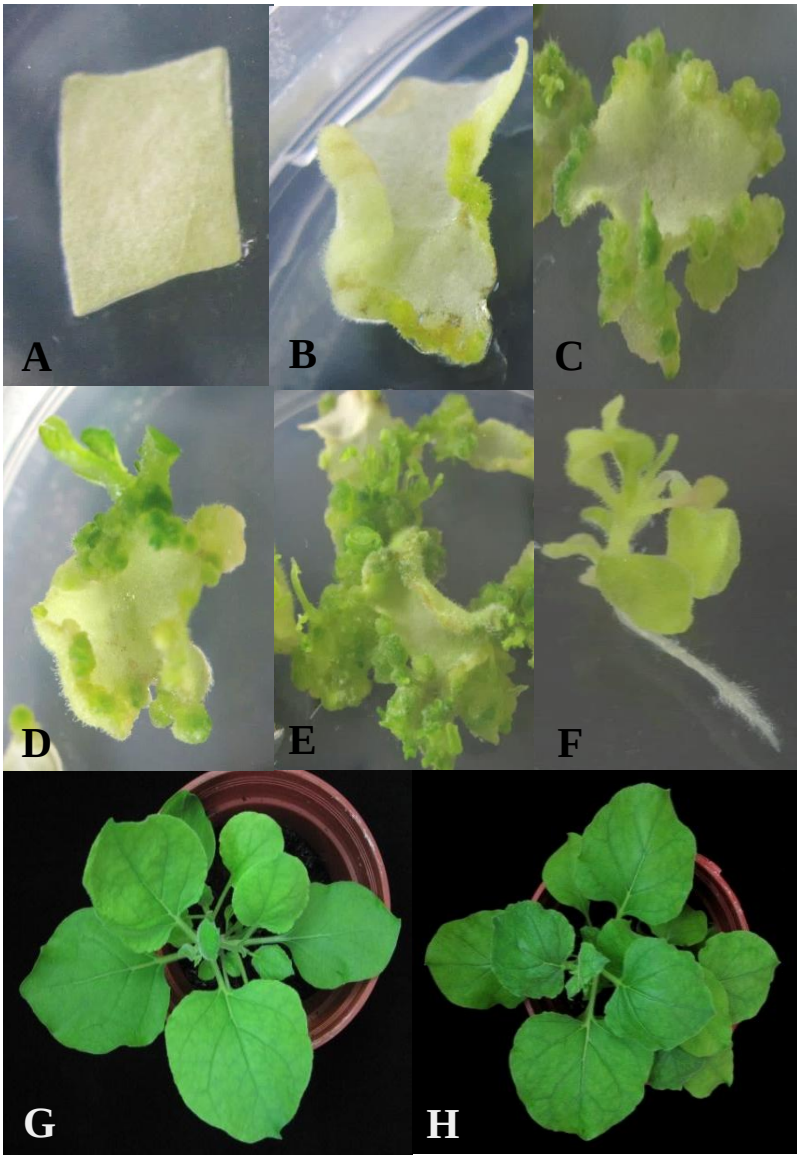

A
B
C
D
E
F
G
H

Figure 3.4 Generation of putative transgenic amiRNA-CymMV-ORSV and antisense-CymMV-ORSV *Nicotiana benthamiana* plants from leaf explants using *Agrobacterium*-mediated transformation.

Leaf explants were co-cultivated in Agrobacteria culture for 30 min before culturing on ½MS plate containing 1 mg/L BA, 0.1 mg/L NAA and 20 mg/L phosphinotricin to induce callus and to screen for transformants **(A)**.

Putative transgenic amiRNA-CymMV-ORSV **(B)** and antisense-CymMV-ORSV **(C)** calli were observed at the edge of the explant.

Putative transgenic amiRNA-CymMV-ORSV **(D)** and antisense-CymMV-ORSV **(E)** shoot differentiated from the calli with other putative transgenic calli formed at the edge of the explant.

Putative transgenic amiRNA-CymMV-ORSV shoot were transferred to fresh ½ MS medium and root formation was observed one month later **(F)**.

Putative transgenic amiRNA-CymMV-ORSV **(G)** and antisense-CymMV-ORSV **(H)** T_0 *N. benthamiana* was able to acclimatise to growing condition.

3.4 Transgene, amiRNA-CymMV-ORSV was PCR-tested in T_0 and T_1 transgenic *N. benthamiana* generated

There were a total of 11 putative T_0 transgenic *N. benthamiana* lines potted into soil, eight putative transgenic lines managed to acclimatised and survived to set seeds. Genomic DNA was extracted from the surviving T_0 putative transgenic *N. benthamiana* lines and PCR was carried out to detect presence of transgene, amiRNA-CymMV-ORSV, in the plant. All the tested T_0 putative transgenic lines were found to contain the transgene (Figure 3.5A).

The T_1 putative transgenic *N. benthamiana* seeds were collected from these six PCR-positive T_0 transgenic lines. Seeds of T_1 putative transgenic lines from each PCR-positive T_0 transgenic lines were sown. Genomic DNA was extracted from eight randomly chosen T_1 putative transgenic lines for detection of transgene presence in the plant. All eight tested T_1 transgenic lines were found to have the transgene (Figure 3.5B), suggesting that the transgene had integrated into the genomic DNA of transgenic *N. benthamiana*. The seeds of the T_1 transgenic lines were collected for detailed analysis of T_2 transgenic plants.

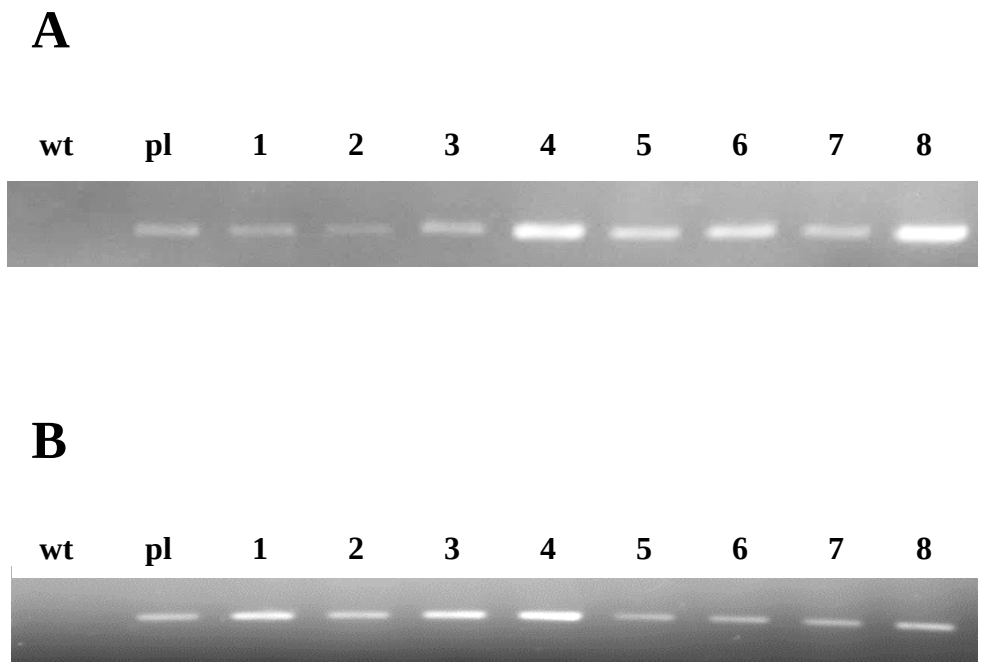


Figure 3.5 Detection of amiRNA-CymMV-ORSV from T_0 and T_1 putative transgenic *Nicotiana benthamiana* lines generated.

Genomic DNA extracted from the putative transgenic **(A)** T_0 and **(B)** T_1 *N. benthamiana* lines were subjected to PCR for detection of transgene, amiRNA-CymMV-ORSV. All the lines tested were PCR-positive.

Lane wt, wild-type *N. benthamiana* as negative control; lane pl, pG0229-oreamiRNA-CymMV-ORSV as positive control; Lanes 1-8, five chosen transgenic *N. benthamiana* lines were tested for presence of transgene and all eight lines showed presence of transgene PCR product.

3.5 Transgene, amiRNA-CymMV-ORSV or antisense-CymMV-ORSV, integrated into the genome of T_2 transgenic *N. benthamiana*

Seeds of T_2 transgenic amiRNA-CymMV-ORSV *N. benthamiana* were sown and genomic DNA was extracted when they are of one-month old. PCR was first performed to detect for presence of transgene, amiRNA-CymMV-ORSV, in the transgenic lines. All seven randomly chosen T_2 transgenic lines were tested to be positive (Figure 3.6A), suggesting that the transgene, amiRNA-CymMV-ORSV, had integrated into the genomic DNA of tested transgenic *N. benthamiana*. Next, T_2 transgenic lines #1 and #2 were chosen for Southern blot analysis. Taken together, these data indicated that the transgene had successfully integrated into the two Southern blot-tested T_2 transgenic lines (Figure 3.6B). The next step was to determine if the transgene, amiRNA-CymMV-ORSV, was able to express in line #1 and line #2.

T_2 transgenic antisense-CymMV-ORSV *N. benthamiana* plants were screened by PCR and Southern blot to determine the integration of the transgene, antisense-CymMV-ORSV, in the plant genome. A total of seven randomly chosen transgenic lines were chosen for PCR screening. All the seven lines were tested to be positive, hinting that the transgene, antisense-CymMV-ORSV, was present in the genome of the transgenic antisense-CymMV-ORSV *N. benthamiana* plants (Figure 3.7A). Subsequently, transgenic line #1 and line 2 were selected for Southern blot analysis. Both transgenic *N. benthamiana* line #1 and line #2 were determined to contain the transgene,

antisense-CymMV-ORSV, as part of its plant genome. Furthermore, line #2 was found to have two copies of the transgene, antisense-CymMV-ORSV, integrated into the plant genome (Figure 3.7B).

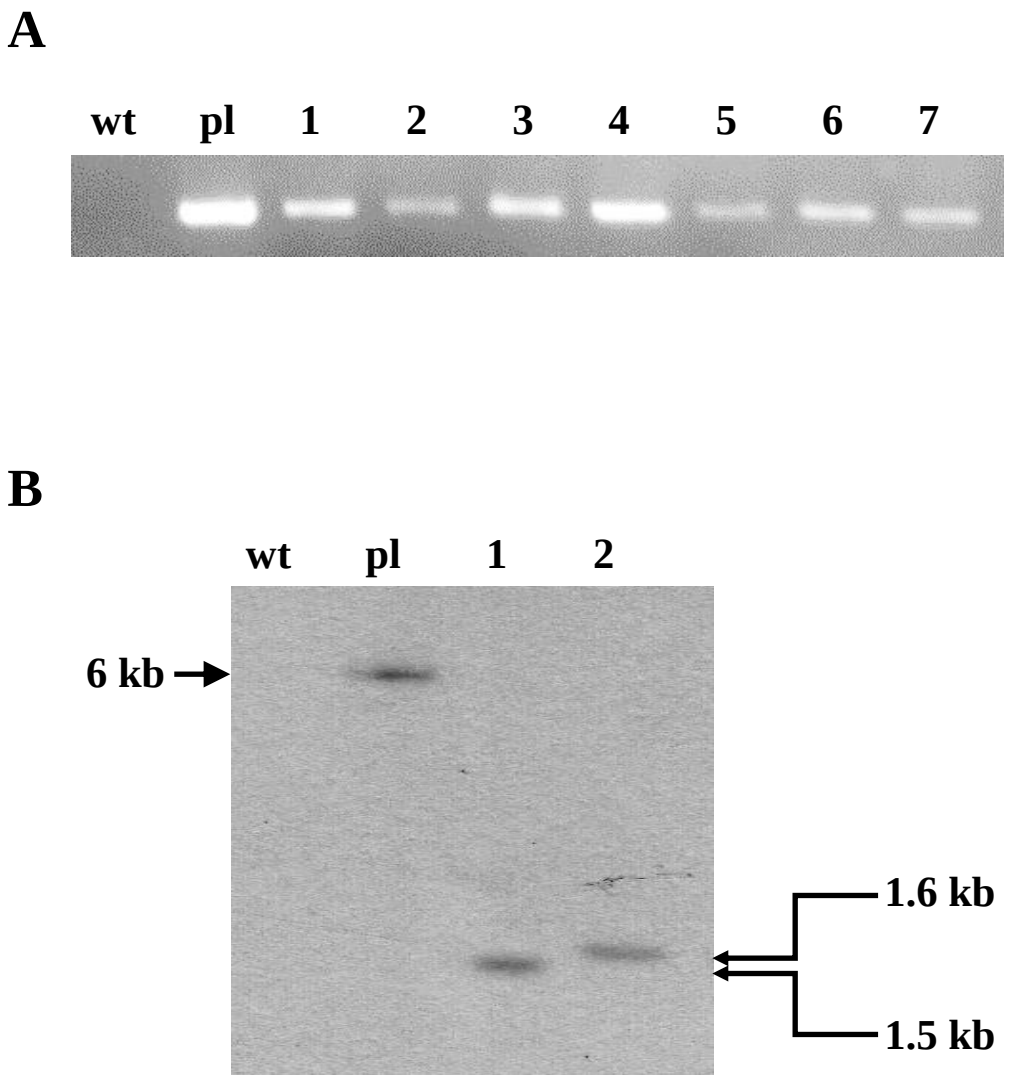


Figure 3.6 Validation of amiR-CymMV-ORSV in putative T_2 transgenic *Nicotiana benthamiana* lines.

(A) Detection of presence of transgene using genomic DNA as template for PCR. The transgene was detected in all randomly chosen seven lines.

(B) Southern blot analysis of line #1 and line #2 from PCR.

Lane wt, wild-type plant as negative control; lane pl, plasmid as positive control; lanes 1-7, selected T_2 lines for testing of transgene presence.

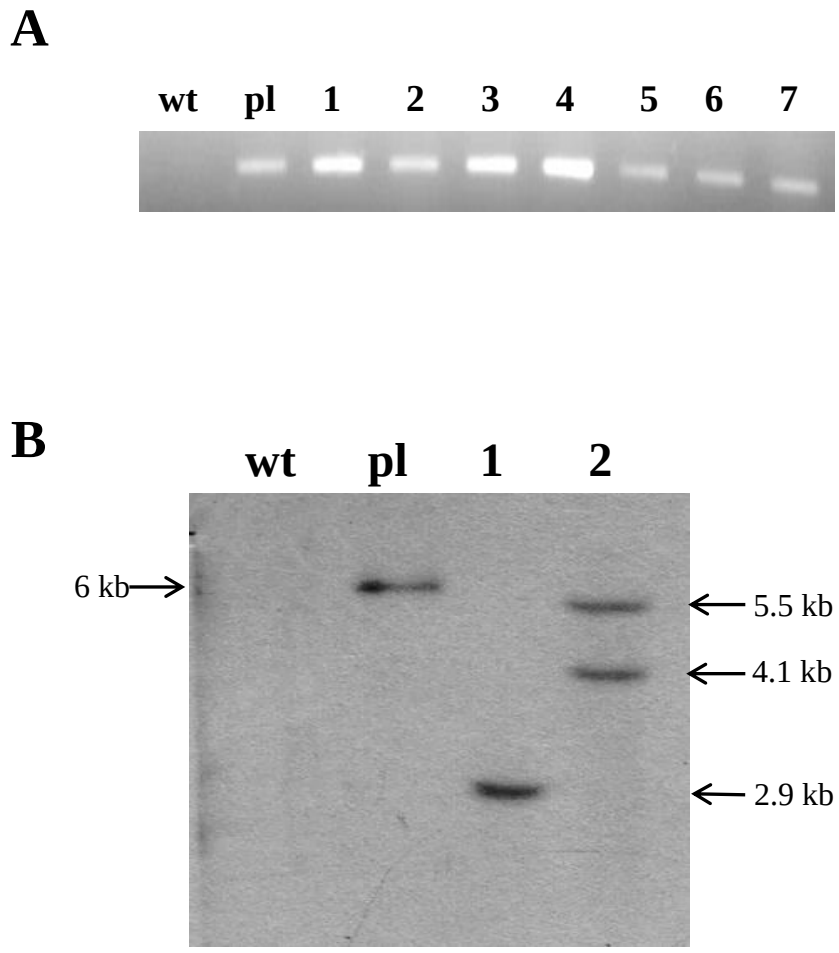


Figure 3.7 Validation of antisense-CymMV-ORSV in putative T_2 transgenic Nicotiana benthamiana lines.

(A) Detection of presence of transgene using genomic DNA as template for PCR. The transgene was detected in all randomly chosen seven lines.

(B) Southern blot analysis of line #1 and line #2 from PCR. Line #2 has two transgene detected.

Lane wt, wild-type plant as negative control; lane pl, plasmid as positive control; lanes 1-7, selected T_2 lines for testing of transgene presence.

3.6 Expression of amiRNA-CymMV-ORSV in T_2 transgenic *N. benthamiana*

The transgene, amiRNA-CymMV-ORSV, was successfully integrated into the genomic DNA of T_2 transgenic amiR-CymMV-ORSV *N. benthamiana*. Next, Northern blot for small RNA was performed to find out if the transgene was able to express in the T_2 transgenic plants. From the two randomly chosen T_2 transgenic plants for small RNA Northern blot analysis, the expression of amiR-CymMV-ORSV was detected for both lines (Figure 3.8). This suggested that amiR-CymMV-ORSV was able to express in the T_2 transgenic amiR-CymMV-ORSV *N. benthamiana* plants.

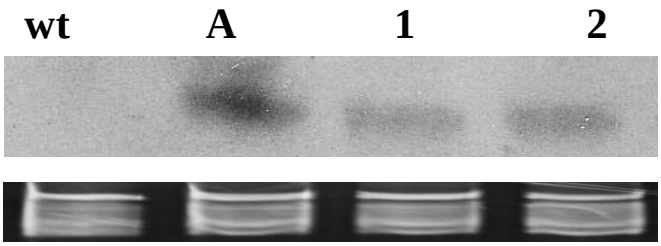


Figure 3.8 Detection of expression of amiR-CymMV-ORSV in T_2 transgenic *Nicotiana benthamiana* lines.

Total RNA was isolated from three different transgenic *N. benthamiana* lines and electrophoresed in a 15% Urea-SDS PAGE gel. Northern blot for small RNA was performed with a 3'- end DIG-labelled probe for detection of viral amiRNA transgene.

Lane wt, wild-type plant as negative control; Lane A, Agroinfiltrated sample as positive control; Lanes 1-2, two T_2 transgenic *N. benthamiana* lines were chosen randomly to check for expression of transgene, amiRNA-CymMV-ORSV. The smaller panel represented the loading control for each sample.

3.8 CymMV and ORSV *in vitro* transcripts were infectious

CymMV *in vitro* transcripts and ORSV *in vitro* transcripts obtained were inoculated onto the leaf of *C. amaranticolor* to check for the infectivity of the viral *in vitro* transcripts. After 10 days post inoculation, local lesions were observed on the leaves inoculated with either CymMV *in vitro* transcripts or ORSV *in vitro* transcripts (Figure 3.9). No local lesion was observed on the leaf inoculated with buffer, suggesting that the local lesions observed were due to the viral *in vitro* transcripts.

After confirming that the viral *in vitro* transcripts were infectious, the next step was to find out if the viral *in vitro* transcripts were able to move within the plants and cause system infection and induce symptoms that were typical after CymMV or ORSV or both CymMV and ORSV infection. An amount of 500 ng of CymMV *in vitro* transcripts or 500 ng of ORSV *in vitro* transcripts or both CymMV and ORSV *in vitro* transcripts were inoculated to *N. benthamiana* plants of six-leaf stage. After 21 days post inoculation, there were intermittent white lines appeared on the leaves inoculated with CymMV *in vitro* transcripts and mild mosaics on leaves and emerging leaves were distorted for plants inoculated with ORSV *in vitro* transcripts. For *N. benthamiana* inoculated with both CymMV and ORSV *in vitro* transcripts, dark green islands appeared on the severe crinkled leaves and the emerging leaves were severely distorted. No disease symptom was observed from mock plants inoculated with buffer (Figure 3.10).

Figure 3.9 Local lesions were induced by *in vitro* transcripts of CymMV and ORSV on *Chenopodium amaranticolor*.

CymMV *in vitro* transcripts and ORSV *in vitro* transcripts was obtained from *in vitro* transcription and they were inoculated onto *C. amaranticolor* to check for its infectivity. The virus *in vitro* transcripts were able to induce local lesion. Mock, leaf inoculated with buffer; CymMV, leaf inoculated with 500 ng of CymMV *in vitro* transcripts; ORSV, leaf inoculated with 500 ng of ORSV *in vitro* transcripts.

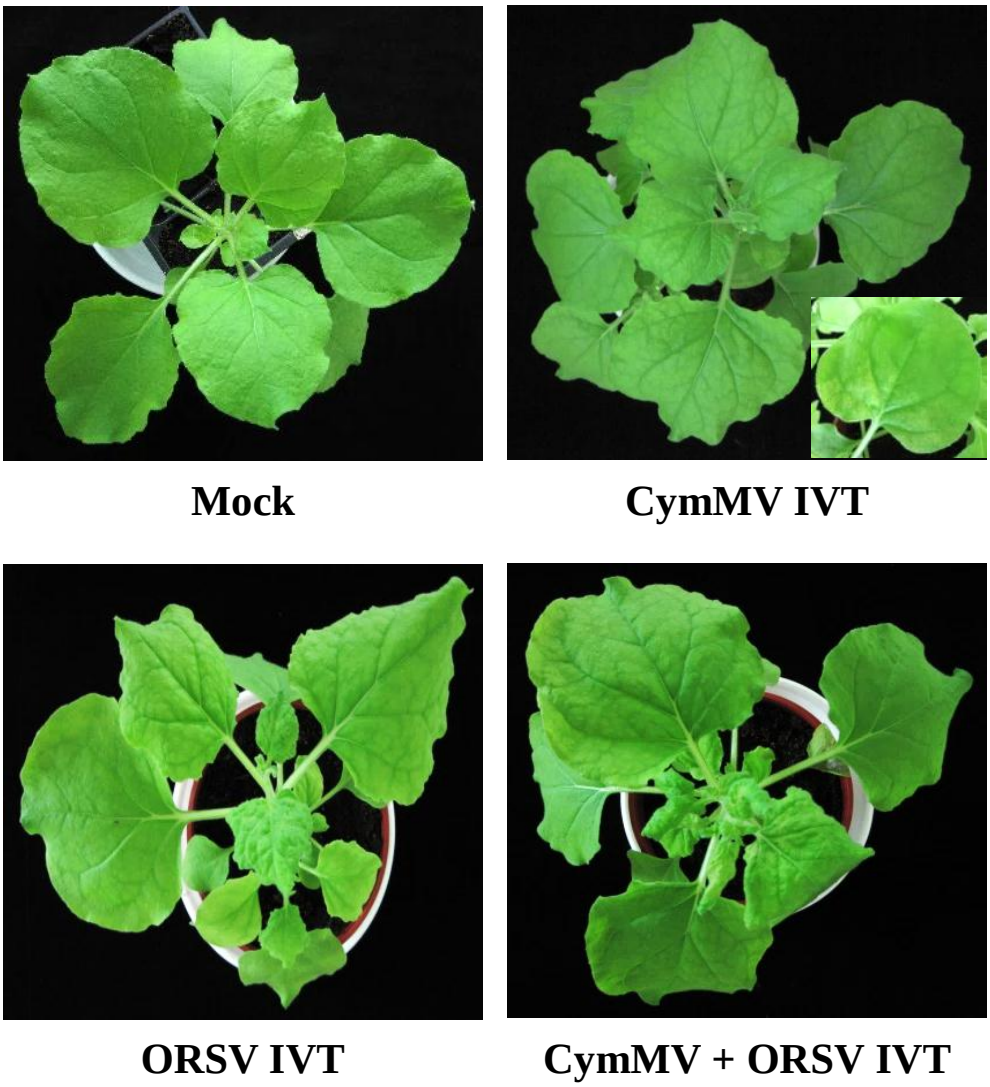


Figure 3.10 Systemic infection was observed after inoculation with *in vitro* transcripts of CymMV, ORSV and CymMV+ORSV on wild-type *Nicotiana benthamiana* plants.

After validated the infectivity of virus *in vitro* transcripts (IVTs) on *C. amaranticolor*, 500 ng of CymMV and ORSV IVTs, singly or doubly were inoculated onto *N. benthamiana* plants to test for its systemic infection ability. Systemic infection was observed 21 days post inoculation on the *N. benthamiana* plants. There were intermittent white lines appeared on the leaves inoculated with CymMV IVTs and mild mosaics on leaves and emerging leaves were distorted for plant inoculated with ORSV IVTs. For *N. benthamiana* inoculated with both CymMV and ORSV *in vitro* transcripts, dark green islands appeared on the severe crinkled leaves and the emerging leaves were severely distorted. Mock plants were treated with buffer as control.

3.8 T_2 transgenic amiR-CymMV-ORSV or antisense-CymMV-ORSV *N. benthamiana* was able to resist against virus challenged

Both CymMV *in vitro* transcripts and ORSV *in vitro transcripts* had proven its infectivity on *C. amaranticolor* and wild-type *N. benthamiana*. The next step was to test the virus resistance capability of the T_2 transgenic *N. benthamiana* plants obtained.

All T_2 transgenic amiR-CymMV-ORSV *N. benthamiana* plants inoculated with CymMV *in vitro* transcripts and ORSV *in vitro* transcripts, singly or doubly, did not show any typical viral disease symptoms after challenged with virus. The virus-inoculated transgenic *N. benthamiana* appeared symptomless and looked similar to wild-type *N. benthamiana* inoculated with buffer as negative control. The experiments were repeated thrice and same phenomenon was observed (Figure 3.11) and 100% of the transgenic plants tested were virus-resistant.

T_2 transgenic antisense-CymMV-ORSV *N. benthamiana* plants were also subjected to inoculation with CymMV *in vitro* transcripts and ORSV *in vitro* transcripts, singly or doubly. The experiments were repeated twice and the transgenic antisense-CymMV-ORSV *N. benthamiana* plants appeared symptomless (Figure 3.12).

RT-PCR and western blot were carried out to detect any presence of viral coat protein gene and coat protein, respectively, in the symptomless T_2 transgenic *N. benthamiana* after virus-inoculation.

No viral coat protein gene was detected from virus-infected transgenic *N. benthamiana* (Figures 3.13 and 3.14). In addition, no viral coat protein was observed from Western blot for virus-infected transgenic *N. benthamiana* (Figures 3.15 and 3.16).

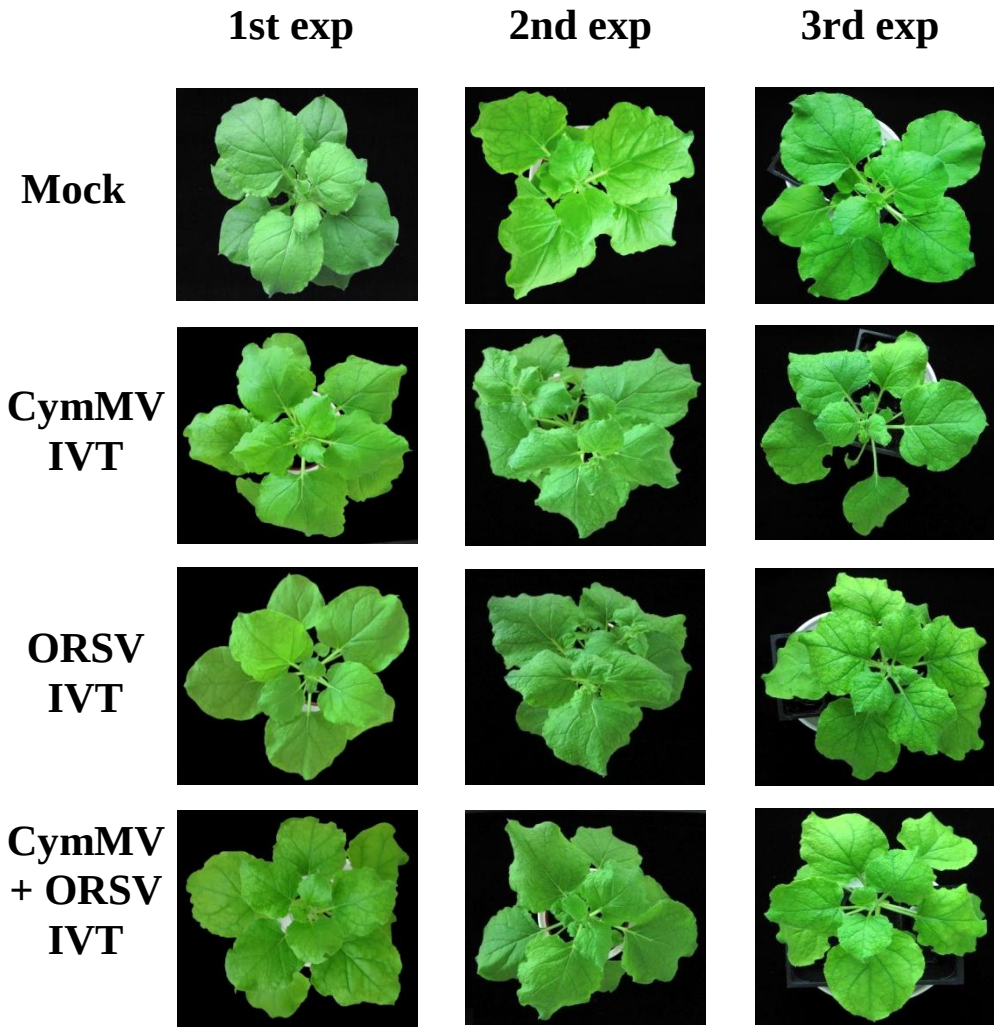


Figure 3.11 Transgenic amiR-CymMV-ORSV *Nicotiana benthamiana* plants were able to resist virus infection.

The virus *in vitro* transcripts (IVT) (500 ng) were inoculated onto transgenic amiR-CymMV-ORSV *N. benthamiana* plants. After 21 days post-inoculation, the transgenic plants did not show any symptoms and they looked similar to mock-treated plant. The experiments (exp) were repeated thrice and the results obtained were similar.

Table 3.1 Summary of number of transgenic amiR-CymMV-ORSV *Nicotiana benthamiana* plants observed with viral symptoms after inoculation with virus *in vitro* transcripts

	Number of plants inoculated with viral symptoms observed			
	Mock	CymMV IVT	ORSV IVT	CymMV+ORSV IVT
Wild-type	0/9	9/9	9/9	9/9
Transgenic	0/9	0/27	0/27	0/27

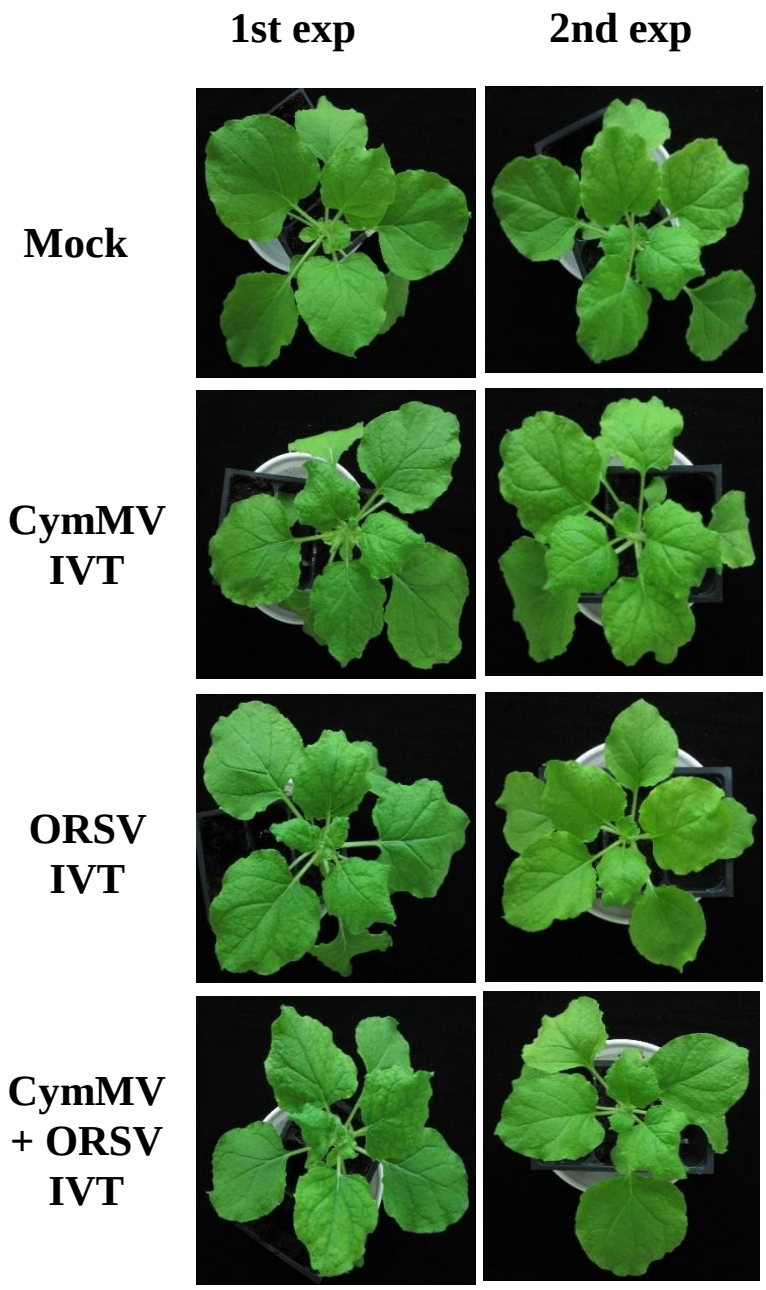
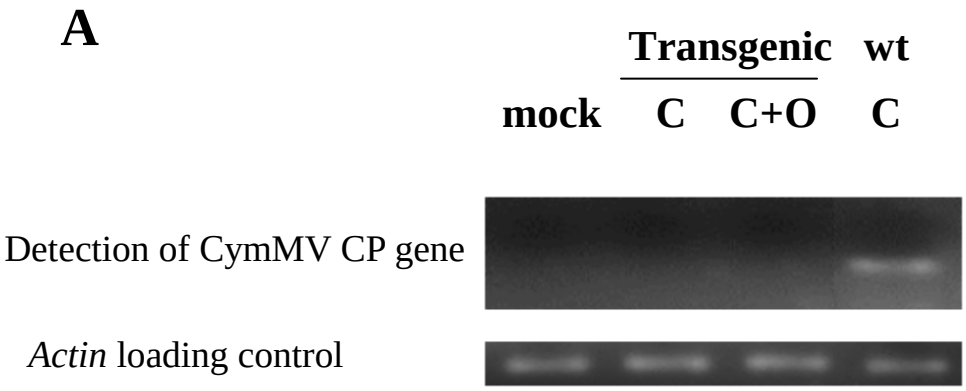


Figure 3.12 Transgenic antisense-CymMV-ORSV *Nicotiana benthamiana* plants were able to resist virus infection.

The virus *in vitro* transcripts (IVT) (500 ng) were inoculated onto transgenic antisense-CymMV-ORSV *N. benthamiana* plants. After 21 days post-inoculation, the transgenic plants did not show any symptoms and they looked similar to mock-treated plant. The experiments (exp) were repeated twice and the results obtained were similar.



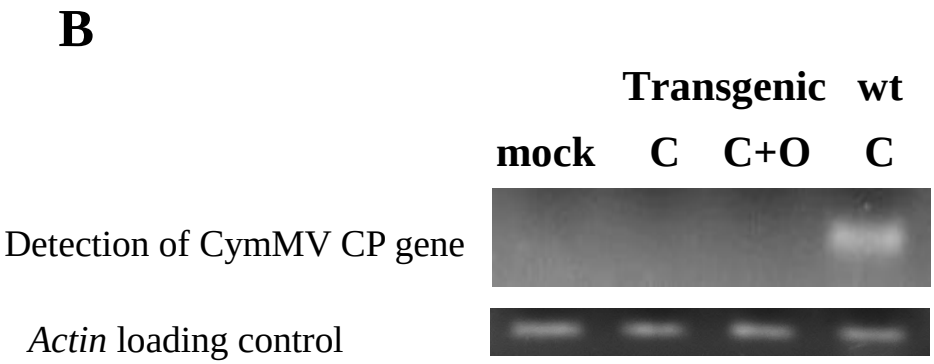


Figure 3.13 Absence of CymMV CP gene in transgenic *Nicotiana benthamiana* lines challenged with *in vitro* transcripts of CymMV or CymMV+ORSV.

Total RNA isolated from transgenic *N. benthamiana* plants infected with CymMV and/or ORSV 21 days post inoculation was used for reverse transcription and its cDNA was used as template in PCR for detection of CymMV CP gene. No CymMV CP gene was detected in virus-infected transgenic amiR-CymMV-ORS **(A)** nor antisense-CymMV-ORS **(B)** *N. benthamiana* plants. PCR products of *actin* were used for loading control.

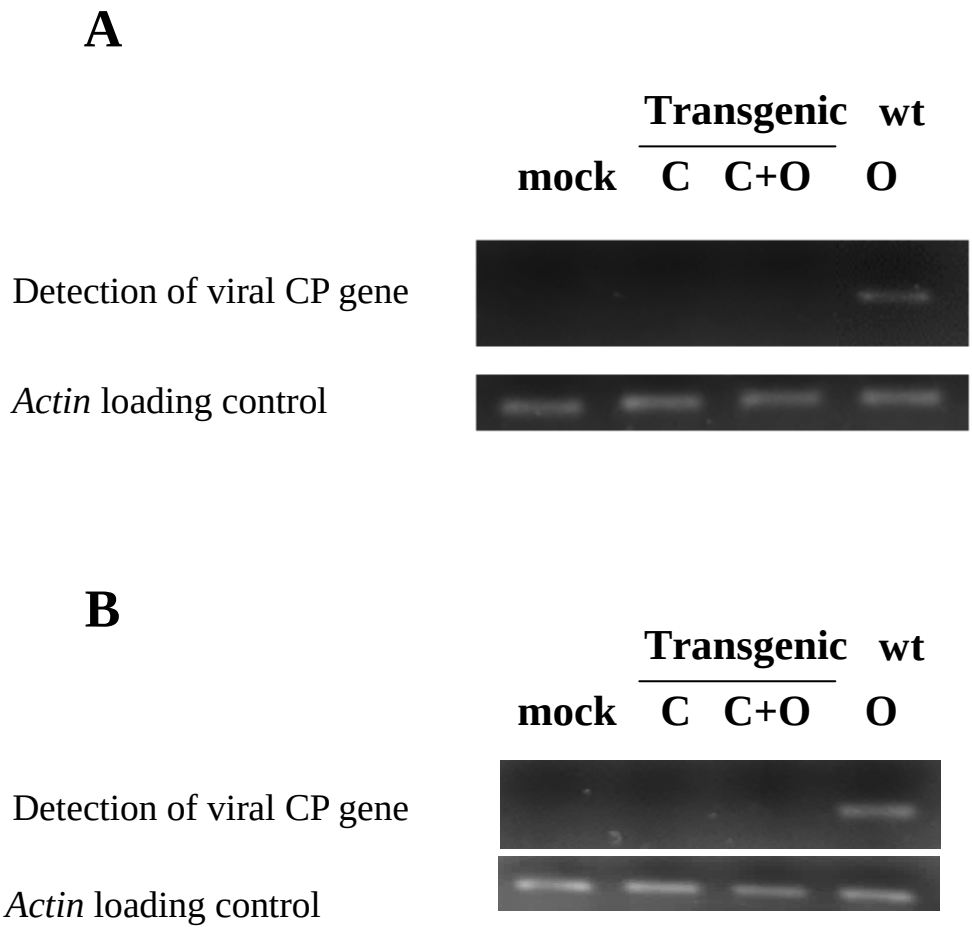


Figure 3.14 ORSV CP gene was not detected in virus-infected transgenic *Nicotiana benthamiana* plants.

Total RNA isolated from transgenic *N. benthamiana* plants infected with CymMV and/or ORSV 21 days post inoculation was used for reverse transcription and its cDNA was used as template in PCR for detection of ORSV CP gene. No ORSV CP gene was detected in virus-infected transgenic amiR-CymMV-ORS **(A)** nor antisense-CymMV-ORS **(B)** *N. benthamiana* plants. PCR products of *actin* were used for loading control.

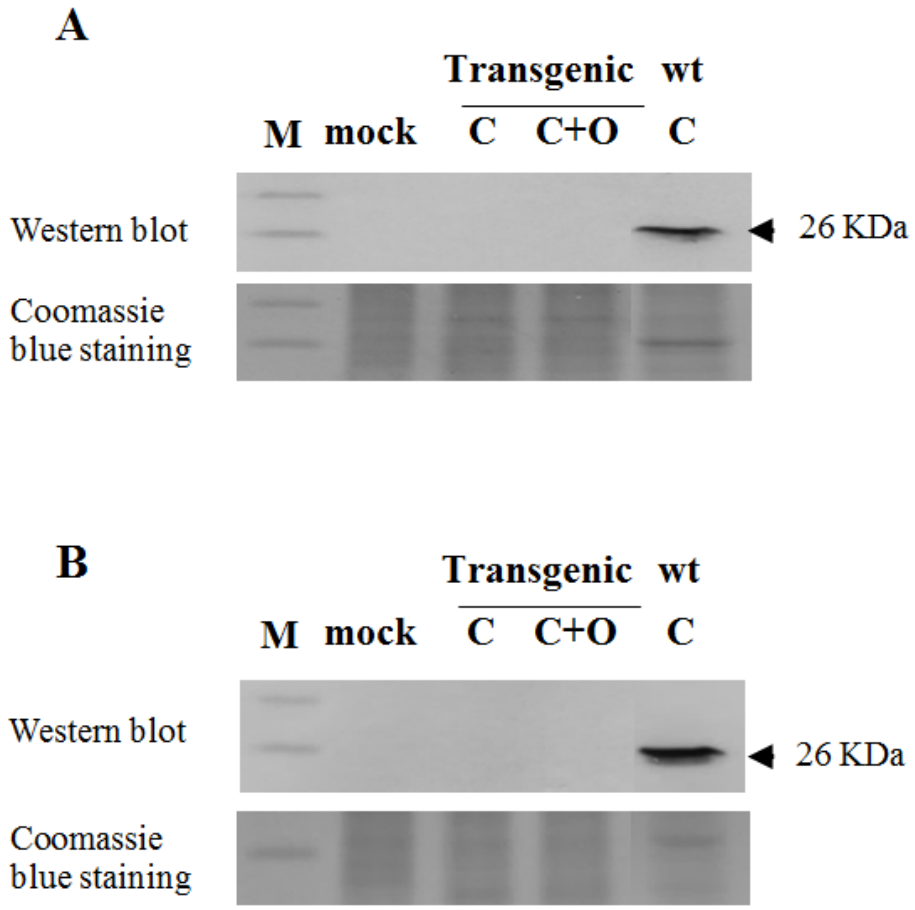


Figure 3.15 No CymMV CP was detected in virus-infected transgenic *Nicotiana benthamiana* 21 days post-inoculation.

Total proteins isolated from transgenic *N. benthamiana* plants infected with CymMV and/or ORSV 21 days post inoculation were used for Western blot and Coomassie blue staining. No CymMV coat protein was detected in virus-infected transgenic amiR-CymMV-ORS **(A)** nor antisense-CymMV-ORS **(B)** *N. benthamiana* plants. Coomassie blue staining was used for loading control.

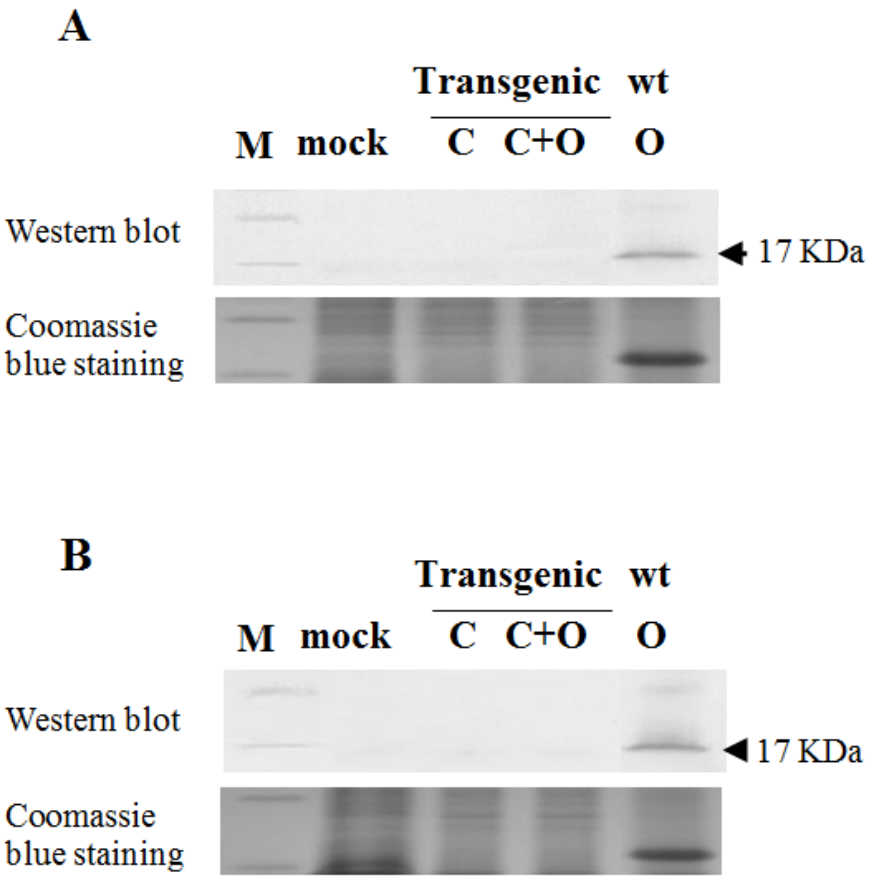


Figure 3.16 No ORSV coat protein was detected in virus-infected transgenic *Nicotiana benthamiana* 21 days post-inoculation.

Total proteins isolated from transgenic *N. benthamiana* plants infected with CymMV and/or ORSV 21 days post inoculation were used for Western blot and Coomassie blue staining. No ORSV coat protein was detected in virus-infected transgenic amiR-CymMV-ORS **(A)** nor antisense-CymMV-ORS **(B)** *N. benthamiana* plants. Coomassie blue staining was used for loading control.

3.9 Attempts to generate transgenic *Dendrobium* Chao Praya Smile with virus resistance

Protocorms were chosen as starting materials for generating transgenic orchids using *Agrobacterium*-mediated transformation approach. Seed capsule of *Dendrobium* Chao Praya Smile was harvested and surface-sterilised. Seeds of *Dendrobium* Chao Praya Smile were released from the capsule onto germination medium and they developed into protocorms. The process from seed capsule development to protocorms stage took five months to achieve (Figure 3.17).

The protocorms were placed in Agrobacteria culture for 3 h and then blotted dried before placing them on selection medium. The putative transformants that grew on the selection medium were subcultured every three weeks to ensure the putative transformants were under the selection stress and to provide nutrients for them to grow on (Figure 3.18).

Genomic DNA was extracted from the leaf tissue of putative transgenic orchid plantlets and it was used for PCR and Southern blot. All four randomly chosen putative transgenic orchid plantlets for detection of transgene showed presence of PCR products similar to sample using plasmid as positive control (Figure 3.19A). Southern blot was performed but no transgene was detected (Figure 3.19B).

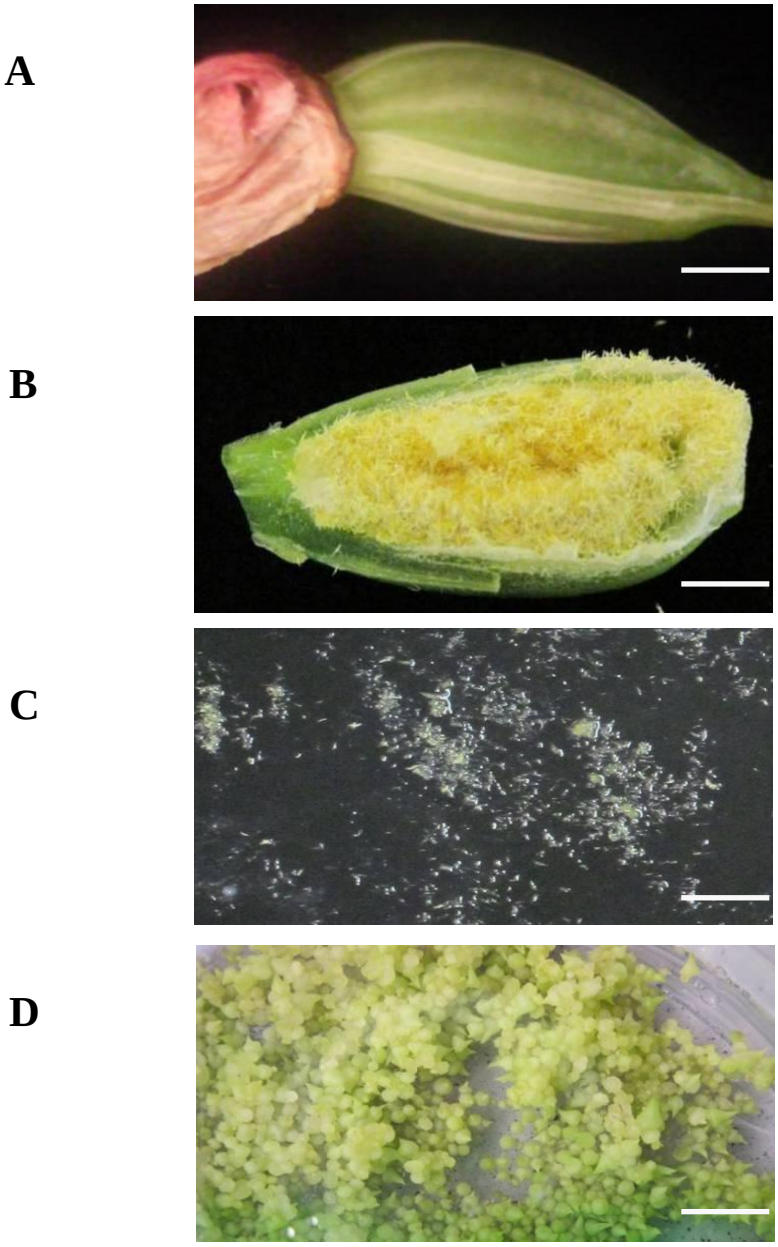


Figure 3.17 Development of *Dendrobium* Chao Praya Smile (DCPS) protocorms from seeds for *Agrobacterium*-mediated transformation.

(A) A three months old DCPS seed pod **(B)** A cross section of the seed pod to reveal the DCPS seeds **(C)** DCPS seeds sown onto germination medium **(D)** Two month-old protocorms on germination medium. The scale bar represented 5 mm.

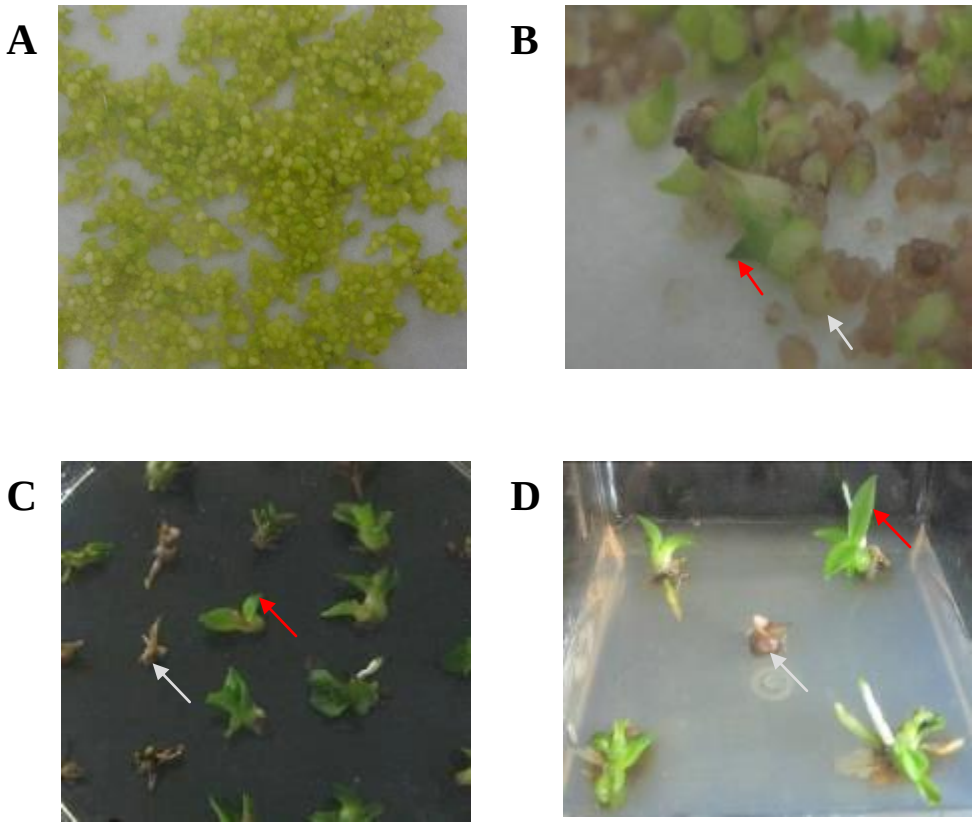


Figure 3.18 Putative transgenic plantlets *Dendrobium* Chao Praya Smile (DCPS) obtained using protocorms subjected to *Agrobacterium*-mediated transformation.

(A) After 3 h of incubating in medium containing Agrobacteria, the protocorms were blotted dry on sterile C-fold towel and placed on KC medium containing 100 μM AS and were placed in dark for three days.

(B) After several rounds of subculturing, several putative transgenic protocorms (red arrowed) managed to survive on KC medium containing 2 μM MSO whereas the unsuccessful transformed protocorms turned necrotic (light grey arrowed).

(C) The surviving putative transgenic protocorms slowly grew into small plantets (red arrowed) and were transferred to fresh KC plates where selection continued to take place. Unsuccessful transformed plantlets did not survive on selection stress.

(D) Putative transgenic orchid plantlets (red arrowed) were subcultured into a GA7 vessel that provided more room for growth. A wild-type DCPS (light grey arrowed) was used as an indicative of selection stress.

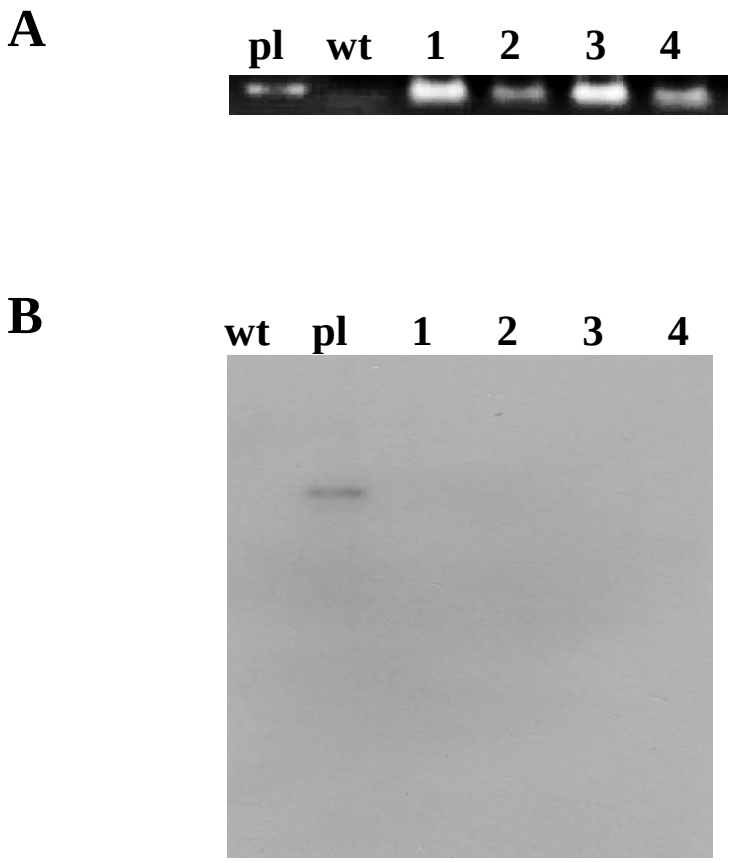


Figure 3.19 Detection of viral artificial microRNA transgene in *Dendrobium* Chao Praya Smile.

(A) Transgene was detected in genomic DNA by PCR.

(B) Southern blot analysis of PCR-tested positive putative transgenic *Dendrobium* Chao Praya Smile lines was not successful.

Lane wt, wild-type plant as negative control; pl, plasmid as positive control; lanes 1-4, putative transgenic orchid samples.

3.10 Pollinia from *Dendrobium* Chao Praya Smile may use as starting materials for orchid transformation

Other than using protocorms, *Dendrobium* Chao Praya Smile pollinia were tested as starting materials towards generating transgenic orchids by *Agrobacterium*-mediated transformation and particle bombardment.

The pollinia were subjected to *Agrobacterium*-mediated transformation by incubating in Agrobacteria culture for 30 min before pollination was performed. Seed capsules were obtained 3 months after pollination (Figure 3.20A). The seeds germinated developed into protocorms. These protocorms were then placed on selection medium for screening of putative transgenic transformants. After continuous subculturing of putative transgenic transformants, they slowly developed into small putative transgenic orchid plantlets surviving on the selection medium (Figure 3.20B). Those unsuccessful transformants failed to grow on the selection medium.

Particle bombardment was carried out using *Dendrobium* Chao Praya Smile pollinia as target tissue. The particle bombardment was performed using slightly squashed pollinia placed on a wetted filter paper (Figure 3.21A). Among the different rupture disc pressure tested, only pollinia subjected to rupture disk pressure of 900 psi and 1550 psi with the distance of stopping screen to the pollinia of 9 and 6 cm, respectively, were able to produce seed capsules (Table 3.2 and Figure 3.21B). Putative transgenic protocorms developed on germination medium containing 0.2 µM of MSO (Figure 3.21D) were transferred to GA7 vessels with more room for growth (Figure 3.21E-H).

A

B

Figure 3.20 Putative transgenic *Dendrobium* Chao Praya Smile (DCPS) plantlets derived from pollinia subjected to *Agrobacterium*-mediated transformation.

(A) Ten-week old developing DCPS seed capsules after pollinated with pollinia incubated with Agrobacteria harbouring pG0229-preamiRNA-CymMV-ORSV. **(B)** Putative transgenic orchid plantlets survived on selection medium (red arrowed) while some orchid plantlets did not survive (white arrowed).

Table 3.2 Summary of seed capsule(s) formed after pollination with bombarded *Dendrobium* Chao Praya Smile pollinia

Other bombardment parameters were the same: the bombardment chamber was evacuated at a pressure of 27 inches Hg; an amount of 3 μg of plasmid DNA was attached to 1 mg of gold particles of 1 nm in diameter.

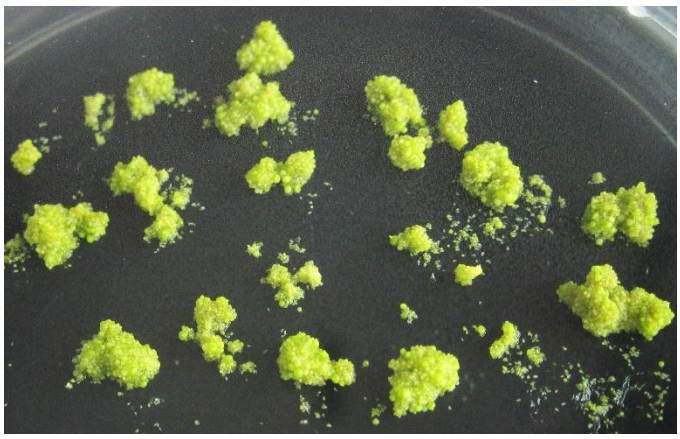
A total of seven flowers were subjected to pollination with bombarded pollinia for each treatment.

Distance of stopping screen to pollinia: 6 cm	
Rupture disk pressure	Total number of seed capsules formed
650	0
900	0
1100	0
1300	0
1550	3
Distance of stopping screen to pollinia: 9 cm	
Rupture disk pressure	Total number of seed capsules formed
650	0
900	2
1100	0
1300	0
1550	0

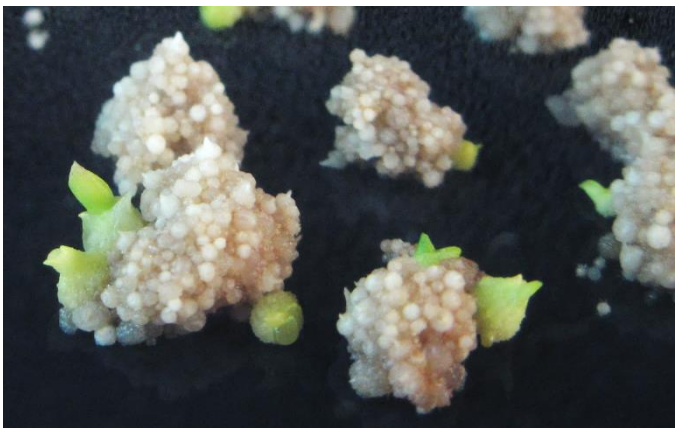
A

B

C



D



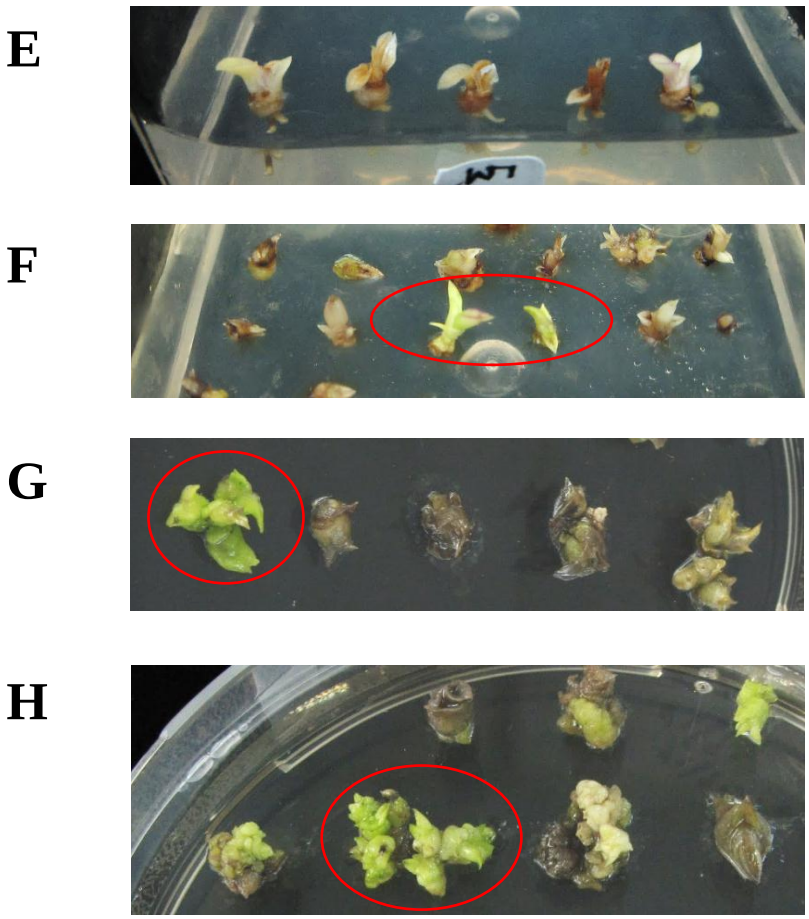


Figure 3.21 Putative transgenic *Dendrobium* Chao Praya Smile (DCPS) small plantlets obtained from pollinia bombarded with gold carrier coated with pG0229-preamiRNA-CymMV-ORSV.

(A) DCPS pollinia were slightly squashed and placed on wet filter paper for particle bombardment. **(B)** Pollination was performed using bombarded pollinia and seed capsule developed indicated pollination was successful. **(C)** Protocorms derived from bombarded pollinia germinated from seeds on the selection medium. **(D)** After two months of selection, minority of putative transgenic protocorms survived on the selection medium. **(E)** Non-transgenic DCPS failed to grow on selection medium. **(F)** Putative transgenic orchid plantlet (red circled) derived from pollinia bombarded under 900 psi treatment. **(G, H)** Putative transgenic orchid plantlet (red circled) derived from pollinia bombarded under 1550 psi treatment.

Chapter 4 Discussion

Orchid flowers quality is affected when viruses like CymMV or ORSV infected the orchids. Orchids that have resistance against viruses are valued by orchid culturists (Chen et al., 2006a). This trait is valued as orchids can be doubly infected with CymMV and ORSV. Over the years, advances in orchid transformation coupled with genetic engineering (Vain, 2006) had gradually set the direction towards generating transgenic orchids. From the reported transgenic orchids, most of them were research-based and geared towards optimization of orchid transformation. There were two reports that used CymMV coat protein to confer virus-resistance on orchids (Liao et al., 2004; Chang et al., 2005) but those transgenic orchids were not seen in the orchid industry. As such, one of the key aspects in this project is to focus towards application-based by attempting to generate transgenic orchids that can resist against CymMV and ORSV infection, eventually aiming to introduce them to the orchid industry.

In this project, amiRNAs strategy was utilized to design viral amiRNAs and target viruses. Given the small size (~22 nt) of amiRNAs, the CymMV and ORSV amiRNAs were placed within a single cassette in this project. This conferred CymMV- and ORSV-resistance to the transgenic plants obtained. This dual virus-resistant strategy used is important as most of the orchid cultivars can be infected by both CymMV and ORSV.

This dual virus-resistant strategy was first verified on transgenic *N. benthamiana* before moving on to generating transgenic orchids. *N.*

benthamiana is a common host for CymMV and ORSV and thus the results obtained can be used as an indicator towards the success of virus-resistant transgenic orchids. CymMV-ORSV-resistant transgenic *N. benthamiana* was generated using *Agrobacterium*-mediated transformation coupling with plant tissue culture techniques. This combination was ideal as Agrobacteria provided a natural-available gene transfer system to introduce and express foreign DNA into plant genome (Chilton et al., 1997) and the totipotency of plant cells allowed the regeneration into a whole plant from the leaf explant.

The transgenic *N. benthamiana* developed demonstrated that the transgene, amiR-CymMV-ORSV can be heritable to future generations as the transgene was detected in T_2 generation, verified by Southern blot. This inferred that viral amiRNAs designed can be stably passed on to the next generation. Furthermore, the normal plant development was observed. This demonstrated that viral amiRNAs chosen can avoid off-target effects (Ossowski et al., 2008). It would be disastrous if off-target silencing occurred as miRNAs are involved in many roles in plants such as development (Kidner and Martienseen, 2005; van Ex et al., 2011; Chen, 2012) and response to abiotic and biotic stress (Sunkar et al., 2007; Lu and Huang, 2008; Lu et al., 2008; Katiyar-Agarwal and Jin, 2010). The viral amiRNAs were able to express normally in the transgenic plant cells as proven by Northern blot. This suggested that the biogenesis of the amiR-CymMV-ORSV was not affected. The virus-resistant capability was proven by using *in vitro* transcripts of CymMV and ORSV as the virus source. *In vitro* transcripts of the two viruses were employed to ensure same amount of virus source was inoculated onto each tested T_2

transgenic *N. benthamiana* plants and the results obtained would be comparable. Based on the results from the transgenic amiR-CymMV-ORSV *N. benthamiana* plants challenged with viruses, it was shown that these transgenic amiR-CymMV-ORSV *N. benthamiana* plants indeed possess the ability to resist CymMV and ORSV, singly or doubly, as 100% of the tested transgenic plants appeared symptomless and no coat protein was detected in the inoculated plants.

Transgenic *N. benthamiana* plants containing the antisense-CymMV-ORSV were obtained through *Agrobacterium*-mediated transformation. They were proven to be effective against CymMV and ORSV, singly or doubly infection.

After proving the feasibility of dual-viral amiRs in a single cassette, amiR-CymMV-ORSV to target specifically CymMV and ORSV, the next step in the project was to work on generating virus-resistant transgenic orchids. By using the protocorms of *Dendrobium* Chao Praya Smile for *Agrobacterium*-mediated transformation, several putative transgenic *Dendrobium* Chao Praya Smile plantlets obtained. Due to the long vegetative state of orchids (Liau et al., 2003a) and the limited time constraint for this project, only molecular analysis via PCR and Southern blot were performed. Transgene, amiR-CymMV-ORSV, was detected by PCR. However, the low amount of genomic DNA extracted was insufficient for Southern blot detection. Currently, there are putative transgenic *Dendrobium* Chao Praya Smile plantlets still surviving on KC medium under MSO selection stress. This observation along with the results gathered from transgenic *N. benthamiana* plants hinted that these putative

transgenic *Dendrobium* Chao Praya Smile plantlets have the ability to resist against CymMV and ORSV.

Another significant result obtained from this project is the potential of using orchid pollinia as starting materials in orchid transformation. Pollinia were not reported to be used as starting materials for orchid transformation. *Dendrobium* Chao Praya Smile pollinia were subjected to either Agrobacterium-mediated transformation or particle bombardment treatment. Both approaches yielded several putative transgenic *Dendrobium* Chao Praya Smile small plantlets as they are still surviving on KC medium under MSO selection stress. As mentioned the limitation of orchid long vegetative state, unfortunately, molecular analysis was unable to be achieved.

Chapter 5 Conclusion and Future Work

The small size of amiRNAs was deliberately utilised by designing and combining CymMV-amiRNA and ORSV-amiRNA into a single cassette and transformed into *A. tumefaciens* GV3101 strain. Transgenic *N. benthamiana* plants were generated from *Agrobacterium*-mediated transformation and T_2 generation was used for virus-resistance analysis. After 21 day post inoculation, all the T_2 transgenic *N. benthamiana* plants inoculated with 500 ng of *in vitro* transcripts of CymMV and ORSV, singly or doubly, displayed disease viral symptomless and no coat protein was detected. This showed that viral amiRNAs approach was successful towards generating virus-resistant transgenic plants with no off-target effects as transgenic *N. benthamiana* plant developed normally as wild-type plants. This is the first case of transgenic plants that can resist against both CymMV and ORSV.

Attempts were made towards generating transgenic *Dendrobium* Chao Praya Smile and several putative transgenic *Dendrobium* Chao Praya Smile plantlets were PCR-tested positive. Due to the long vegetative state of orchids, and time frame given for the project, further molecular analysis was unfortunately unable to proceed. But the putative transgenic *Dendrobium* Chao Praya Smile plantlets are still growing on KC medium and surviving under MSO selection stress, along with the data from T_2 transgenic *N. benthamiana*; these putative transgenic orchids possess the ability to resist against CymMV and ORSV infection.

A significant finding in this project is the potential of using pollinia as starting materials in orchid transformation. From the results obtained, several putative small *Dendrobium* Chao Praya Smile plantlets are growing on KC medium with MSO selection stress in place, it is speculated that pollinia could be used in orchid transformation.

There are several experiments in place for future work:

1a. Molecular analysis needs to be performed to validate the putative transgenic *Dendrobium* Chao Praya Smile plantlets obtained in this project, which are still growing on KC medium with MSO.

1b. Once validation process is completed, the transgenic orchid will be subjected to virus challenge to determine the virus resistance capability.

2. The transgenic orchids can then be marketed to the orchid industry. Concurrently, the stability and heritability of the amiR-CymMV-ORSV transgene can be tested by using *in vitro* flowering (Hee et al., 2007) where the flowering period is reduced to 1 year from 3-5 years.

3. Viral *in vitro* transcripts of 500 ng was inoculated to each tested transgenic T_2 *N. benthamiana* plants. All the tested transgenic T_2 *N. benthamiana* plants showed resistance to CymMV and/or ORSV infection. It would be interesting to find out if these transgenic T_2 *N. benthamiana* plants were still able to resist against virus infection when the amount of *in vitro* transcripts inoculated is increased to 1000 ng or 1500 ng. The hypothesis that comes into mind is if not all transgenic T_2 *N. benthamiana* plants were able to resist against higher

amount of viral *in vitro* transcripts inoculated, does the expression level of amiR-CymMV-ORSV plays a role?

4. Other than virus-resistant trait valued by orchid culturists, the characteristics of flowers like early flowering or unique colours or shapes are being sought after. With amiRNAs strategy, multiple amiRNAs targeting different virus genome region, different viruses, flowering time can all be incorporated into a single cassette. This saves the hassle of multiple transformations, which is a challenge when it comes to orchid transformation.

In conclusion, orchids play a valuable role in floriculture world-wide. It is important to apply new genetic engineering and plant transformation methods to generate orchids with different unique desirable characteristics to meet the demands of consumers, and floriculturists and orchid industry players. This project had produced transgenic plants using amiRNAs strategy to target both CymMV and ORSV and had shown the potential of using orchid pollinia as starting materials for orchid transformation.

References

Aalto, A., and Pasquinelli, A. 2012. Small non-coding RNAs mount a silent revolution in gene expression. Current Opinion in Cell Biology 24:333-340.

Abel, P., Nelson, R., De, B., Hoffmann, N., Rogers, S., Fralev, R., and Beachy, R.N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.

Ai, T., Zhang, L., Gao, Z., Zhu, C.X., and Guo, X. 2010. Highly efficient virus resistance mediated by artificial microRNAs that target the suppressor of PVX and PVY in plants. Plant Biology 80:2429-2436.

Ajjikuttira, P. (2003). Genetic variability and interactions of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* (Singapore: National University of Singapore).

Alonso, E., Garcia-Luqus, I., de la Cruz, A., Wicke, B., Avila-Rincom, M., Serra, M., Castresana, C., and Diaz-Ruiz, J. 1991. Nucleotide sequence of the genomic RNA of pepper mild mottle virus, a resistance-breaking tobamovirus in pepper. The Journal of General Virology 72:2875-2884.

Anzai, H., Ishii, Y., Shichinohe, M., Katsumata, K., Nojiri, C., Morikawa, H., and Tanaka, M. 1996. Transformation of *Phalaenopsis* by particle bombardment. Plant Tissue Culture Letters 13:265-272.

Arditti, J. 1992. Fundamentals of orchid biology. John Wiley & Sons Inc, New York.

Baker, B., Zambryski, P., Staskawicz, B., and Dinesh-Kumar, S. 1997. Signaling in plant-microbe interactions. Science 276:726-733.

Baker, C., and Baker, M. 1996. Orchid Species Culture: *Dendrobium*. Timber Press Inc.

Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281-297.

Beachy, R.N., Loesch-Fries, S., and Tumer, N. 1990. Coat protein-mediated resistance against virus infection. Annual Review of Phytopathology 28:451-474.

Belarmino, M., and Mii, M. 2000. *Agrobacterium*-mediated genetic transformation of a *Phalaenopsis* orchid. Plant Cell Reports 19:435-442.

Binns, A. 1990. *Agrobacterium*-mediated gene delivery and the biology of host range limitations. Physiologia Plantarum 79:135-139.

Binns, A., and Thomashow, M. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. Annual Review of Microbiology 42:575-606.

Birch, R. 1997. Plant transformation: Problems and strategies for practical application. Annual Review of Plant Pathology and Plant Molecular Biology 48:297-326.

Block, M., Brouwer, D., and Tenning, P. 1989. Transformation of *Brassica napus* and *Brassica oleracea u*sing *Agrobacterium tumefaciens* and the expression of the bar and neo genes in the transgenic plants. Plant physiology 91:694-701.

Blumenstiel, J. 2011. Evolutionary dynamics of transposable elements in a small RNA world. Trends in Genetics 27:23-31.

Bolton, G., Nestor, E., and Gordon, M. 1986. Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. Science 232:983-985.

Brown, D., Robertson, W., and Trudgill, D. 1995. Transmission of viruses by plant nematodes. Annual Review of Phytopathology 33:223-249.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C., Damania, B., and Cullen, B.R. 2005. Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. Proceedings of the National Academy of Sciences of the United States of America 102:5570-5575.

Callaway, A., Glesman-Cookmeyer, D., Gillock, E., Sit, T., and Lommel, S. 2001. The multifunctional capsid proteins of plant RNA viruses. Annual Review of Phytophathology 39:419-460.

Campbell, R. 1996. Fungal transmission of plant viruses. Annual Review of Phytopathology 34:87-108.

Carrington, J., Kasschau, K., Mahajan, S., and Schaad, M. 1996. Cell-to-cell and long-distance transport of viruses in plants. The Plant Cell 8:1669-1681.

Chai, D., Lee, S., Ng, J., and Yu, H. 2007. L-Methionine sulfoximine as a novel selection agent for genetic transformation of orchids. Journal of Biotechnology 131:466-472.

Chai, M., Xu, C., Senthil, K., Kim, J., and Kim, D. 2002. Stable transformation of protocorm-like bodies in *Phalaenopsis* orchid mediated by *Agrobacterium tumefaciens*. Scientia Horticulturae 96:213-224.

Chan, M., Chan, Y., and Sanjaya. 2006a. Orchids (*Cymbidium* spp., *Oncidium* and *Phalainopsis*). Pages 331-337 in: Methods in Molecular Biology, Vol. 344: Agrobacterium *Protocols.* 2/e, K. Wang, ed. Humana Press Inc, Totowa, USA.

Chan, M., Sanjaya, and Teixeira da Silva, J.A. 2006b. *Oncidiumi* tissue culture, transgenic and biotechnology. Pages 193-198 in: Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues, J.A. Teixeira da Silva, ed. Global Science Books, Ltd., London, UK.

Chan, Y.-L., Lin, K.-H., Sanjaya, Liao, L.-J., Chen, W.-H., and Chan, M.-T. 2005a. Gene stacking in *Phalaenopsis* orchid enhances dual tolerance to pathogen attack. Transgenic Research 14:279-288.

Chan, Y., Lin, K., Sanjaya, Liao, L.J., Chen, W.H., and Chan, M.T. 2005b. Gene stacking in *Phalaenopsis* orchid enhances dual tolerance to pathogen attack. Transgenic Research 14:279-288.

Chang, C., Chen, Y., Hsu, Y., Wu, J., Hu, C., Chang, W., and Lin, N. 2005. Transgenic resistance to *Cymbidium mosaic virus* in *Dendrobium* expressing the viral capsid protein gene. Transgenic Research 14:41-46.

Chapman, E., and Carrington, J.C. 2007. Specialization and evolution of endogenous small RNA pathways. Nature Reviews Genetics 8:884-896.

Chen, X. 2010. Small RNAs - secrets and surprises of the genome. The Plant journal : for cell and molecular biology 61:941-958.

Chen, X. 2012. Small RNAs in development - insight from plants. Current Opinion in Genetics & Development 22:361-367.

Cheng, Z., Schnurr, J., and Kapaun, J. 1998. Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. Plant Cell Reports 17:646-649.

Chia, T., Chan, Y., and Chua, N. 1992. Characterisation of *Cymbidium mosaic virus* coat protein gene and its expression in transgenic tobacco plants. Plamt Molecular Biology 18:1091-1099.

Chia, T., Chan, Y., and Chua, N. 1994. The firefly luciferase gene as a noninvasive reporter for *Dendrobium* transformation. The Plant journal : for cell and molecular biology 6:441-446.

Chilton, M., Drummond, M., Merlo, D., Sciaky, D., Montoya, A., Gordon, M., and Nester, E. 1977. Stable incorporation of plasmid DNA into higher plant cells: The molecular basis of crown gall tumorigenesis. Cell 11:263-271.

Chin, D., Mishiba, K., and Mii, M. 2007. *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cymbidium*. Plant Cell Reports 26:735-743.

Chng, C., Wong, S., Mahtani, P., Loh, C., Goh, C., Kao, M., Chung, M., and Watanabe, Y. 1996. The complete sequence of a Singapore isolate of *Odontoglossum ringspot virus* and comparison with other tobamoviruses. Gene 171:155-161.

Christou, P. 1994. Particle gun mediated transformation. Current Opinion in Biotechnology 4:135-141.

Culver, J., and Padmanabhan, M. 2007. Virus-induced disease: altering host physiology one interaction at a time. Annual Review of Phytophathology 45:221-243.

Cuozzo, M., O'Connell, K., Kaniewski, J., Fang, R., Chua, N.H., and Tumer, N. 1988. Viral protection in transgenic tobacco plants expressing the Cucumber mosaic virus coat protein or its antisense RNA. Nature Biotechnolgy 6:549-557.

Damsteegt, V., Scorza, R., Gidow, F., Schneider, W., Stone, A., and Luster, D. 2004. Comparative host range of US isolates of Plum pox virus among Prunus and other woody plant species following graft inoculation or aphid transmission. Phytopathology 94.

Ding, L., Wang, Y., and Yu, H. 2013. Overexpression of *DOSOC1*, an ortholog of Arabidopsis *SOC1*, promotes flowering in the orchid *Dendrobium* Chao Praya Smile. Plant and Cell Physiology 54:595-608.

Duan, C., Wang, C.H., and Guo, H.S. 2012. Application of RNA silencing to plant disease resistance. Science 3:5.

Elbashir, S., Lendecker, W., and Tuschl, T. 2001a. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes & development 15:188-200.

Elbashir, S., Harborth, J., Lendecker, W., Yalcin, A., Webber, K., and Tuschl, T. 2001b. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494-498.

Evans, R., Haley, B., and Roth, D. 1985. Photoaffinity labelling of a viral induced protein from tobacco. Journal of Biological Chemistry 260:7800-7804.

Fire, A., Xu, S., Montgomery, M., Kostas, S., Driver , S., and Mello, C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806-811.

Francki, R. 1970. *Cymbidium mosaic virus*. in: Descriptions of Plant Viruses No. 27, CMI/AAB, Wellesbourne, Warwick.

Gelvin, S.B., and Liu, C. 1994. Genetic manipulation of *Agrobacterium tumefaciens* strains to improve transformation of recalcitrant species. Pages 85-97 in: Plant Molecular Biology Manual, S.B. Gelvin and R. Schilperoort, eds. Springer Netherlands.

Glick, E., Zrachya, A., Levy, Y., Mett, A., Gidoni, D., Belausov, E., Citovsky, V., and Gafni, Y. 2008. Interaction with host SGS3 is required for suppression of RNA silencing by Tomato yellow leaf curl virus V2 protein. Proceedings of the National Academy of Sciences of the United States of America 105:157-161.

Goorbalenya, A., and Koonin, R. 1989. Viral proteins containing the purine NTP-binding sequence pattern. Nucleic Acids Research 17:7735-7762.

Habili, N., and Symons, R. 1989. Evolutionary relationship between luteoviruses an other RNA plant viruses based on sequence motifs in their putative RNA polymerases and nucleic acid helicases. Nucleic Acids Research 17:9543-9555.

Hamilton, A., and Baulcombe, D. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950-952.

Hammerschlag, F., Zimmerman, R., Yadava, U., Hunsucker, S., and Gercheva, P. 1997. Effect of antibiotics and exposure to an acidified medium on the elimination of *Agrobacterium tumefaciens* from apple leaf explants and on shoot regeneration. Journal of the American Society for Horticulture Science 112:758-763.

He, X., Fang, Y., Feng, L., and Guo, H. 2008. Characterization of conserved and novel microRNAs and their targets, including a TuMV-induced TIR-NBS-LRR class R gene-derived novel miRNA in Brassica. FEBS Letters 582:2445-2452.

Heaton, L., Lee, T., Wei, N., and Morris, T. 1991. Point mutations in the turnip crinkle virus capsid protein affect the symptoms expressed by *Nicotiana benthamiana*. Virology 183:143-150.

Hee, K.H., Loh, C.S., and Yeoh, H.H. 2007. Early in vitro flowering and seed production in culture in *Dendrobium* Chao Praya Smile (Orchidaceae). Plant Cell Reports 26:2055-2062.

Helliwell, C.A., and Waterhouse, P.M. 2005. Constructs and methods for hairpin RNA-mediated gene silencing in plants. Methods in Enzymology 392:24-35.

Hemenway, C., Fang, R., Kaniewski, J., Chua, N., and Tumer, N. 1988. Analysis o f the mechanism of protection in transgenic plants expressing the Potato virus X coat protein or its antisense RNA. The EMBO Journal 7:1273-1280.

Hiei, Y., Komari, T., and Kuba, T. 1997. Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Molecular Biology 35:205-218.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. The Plant journal : for cell and molecular biology 6:271-282.

Hooykaas, P., and Schilperoort, R. 1992. *Agroacterium* and Plant genetic engineering. Plant Molecular Biology 19:15-38.

Hsieh, R., Chen, W.H., Hsu, H., Lin, Y., Tsai, W., Fu, Y., Chan, M.T., and Yu, S. 1997. *Agrobacterium tumefaciens*-mediated transformation of *Phalaenopsis* orchid. Annual Report - Taiwan Sugar Research Institute 155:41-54.

Hsieh, Y., and Huang, P. 1995. Studies on genetic transformation of *Phalaenopsis* via pollen tube pathway. Journal of the Chinese Society Horticultural Science 41:309-325.

Huijberts, N., Blystad, D., and Bos, L. 1990. Lettuce big-vein virus - mechanical transmission and relationships to Tobacco stunt virus. Annals of Applied Biology 116:463-475.

Hull, R. 2013. Plant Virology. Academic Press, Norwich, U.K.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T., and Kumashiro, T. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnolgy 14:745-750.

Jensen, D., and Gold, A. 1951. A virus ringspot of odontoglossum orchid: symptoms, transmission and electron microscopy. Phytopathology 41:648-653.

Jones-Rhoades, M., Bartel, D.P., and Bartel, B. 2006. MicroRNAs and their regulatory roles in plants. Annual Review of Plant Biology 57:19-53.

Kanchroo, P., -Shekara, A.C., and Klessig, D. 2006. Plant signal transduction and defense against viral pathogens. Advances in Virus Research 66:161-191.

Katiyar-Agarwal, S., and Jin, H. 2010. Role of small RNAs in host-microbe interactions. Annual Review of Phytopathology 48:225-246.

Khentry, Y., Chowpongpang, S., Paradornuwat, A., Tantiwiwat, S., and Thaveechai, N. 2007. Detection of *Cymbidium mosaic virus* in protocorm-like bodies in *Dendrobium* sonia using one-step RT-PCR. Journal of Plant Biochemistry and Biotechnology 16:123-125.

Kidner, C., and Martienseen, R. 2005. The developmental role of microRNA in plants. Current Opinion in Plant Biology 8:38-44.

Klein, T., Wolf, E., Wu, R., and Sanford, J. 1987. High-velocity of microprojectile for delivering nucleic acids into living cells. Nature 327:70-73.

Klein, T., Fromm, M., Weissinger, A., Tomes, D., Schaaf, S., Sletten, M., and Sanford, J. 1988. Transfer of foreign genes into intact maize cells with high velocity microprojectiles. Proceedings of the National Academy of Sciences of the United States of America 85:4305-4309.

Knapp, J., Kausch, A., and Chandlee, J. 2000. Transformation of three genera of orchid using the bar gene as a selectable marker. Plant Cell Reports 19:893-898.

Koch, R. 1893. Über den augenblicklichen Stand der bakteriologischen Choleradiagnose. Zeitschrift für Hygiene und Infectionskrankheiten 14:319-333.

Komari, T., Hiei, Y., Ishida, Y., Kumashiro, T., and Kubo, T. 1998. Advances in cereal gene transfer. Current Opinion in Plant Biology 1:161-165.

Kuehnle, A., and Sugii, N. 1992. Transformation of *Dendrobium* orchid using particle bombardment of protocormss. Plant Cell Reports 11:484-488.

Kung, S., and Yang, S. 1998. Discoveries in Plant Biology. World Publishing Co., Hong Kong.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E.J., Carrington, J.C., Liu, Y., Dolja, V., Calvino, L., López-Moya, J., and Burgyán, J. 2006. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. The EMBO Journal 25:2768-2780.

Lawson, C., Kaniewski, W., Haley, L., Rozman, R., Newell, C., Sanders, P., and Tumer, N. 1990. Engineering resistance to mixed virus infection in a commercial potato cultivar: Resistance to Potato virus X and Potato virus Y in transgenic Russet Burbank. Nature Biotechnolgy 8:127-134.

Lawson, R., and Brannigan, M. 1986. Virus diseases of orchids. in: Handbook of Orchid Pests and Diseases, American Orchid Society, West Palm Beach, Florida.

Li, S., Kuoh, C., Chen, Y., Chen, H., and Chen, W. 2005. Osmotic sucrose enhancement of single-cell embryogenesis and transformation

efficiency in *Oncidium*. Plant Cell, Tissue and Organ Culture 81:183-192.

Liao, L., Pan, I., Chan, Y., Hsu, Y., Chen, W., and Chan, M. 2004. Transgene silencing in *Phalaenopsis* expressing the coat protein of *Cymbidium mosaic virus* is a manifestation of RNA-mediated resistance. Molecular Breeding 13:229-234.

Liau, C., You, S., Prasad, V., Hsiao, H., Lu, J., Yang, N., and Chan, M. 2003a. *Agrobacterium tumefaciens*-mediated transformation of an *Oncidium* orchid. Plant Cell Reports 21:993-998.

Liau, C.H., Lu, C., Prasad, V., Hsiao, H.H., You, S.J., Lee, J., Yang, N.S., Huang, H., Feng, T., Chen, W.H., and Chan, M.T. 2003b. The sweet pepper ferredoxin-like protein (pflp) conferred resistance against soft rot disease in *Oncidium* orchid. Transgenic Research 12:329-336.

Lindsey, K. 1992. Genetic manupulation of crop plants. Journal of Biotechnology 26:1-28.

Liu, Q., and Chen, Y. 2010. A new mechanism in plant engineering: the potential roles of microRNAs in molecular breeding for crop improvement. Biotechnology Advances 28:301-307.

Loesch-Fries, S., Merlo, D., Zinnen, T., Burhop, L., Hall, K., Krahn, K., Jarvis, N., Nelson, S., and Halk, E. 1987. Expression of Alfalfa mosaic virus RNA 4 in transgenic plants confers virus resistance. The EMBO Journal 6:1845-1851.

Lomonossoff, G. 1995. Pathogen-derived resistance to plant viruses. Annual Review of Phytopathology 33:323-343.

Lu, R., Martin-Hernandez, A., Peart, J., Malcuit, I., and Baulcombe, D. 2003. Virus-induced gene silencing in plants. Methods 30:296-303.

Lu, X., and Huang, X. 2008. Plant miRNAs and abiotic stress responses. Biochemical and Biophysical Research Communications 368:458-462.

Lu, Y., Gan, Q., Chi, X., and Qin, S. 2008. Roles of microRNA in plant defense and virus offense interaction. Plant Cell Reports 27:1571-1579.

Lucas, W. 2006. Plant viral movement proteins: Agents for cell-to-cell trafficking of viral genomes. Virology 344:169-184.

Lucas, W., and Gilbertson, R. 1994. Plasmodesmata in relation to viral movement within leaf tissues. Annual Review of Phytophathology 32:387-422.

Maughan, S., and Cobbett, C. 2003. Methionine sulfoximine, an alternative selection for the bar marker in plants. Journal of Biotechnology 102:125-128.

Men, S., Ming, X., Liu, R., Wei, C., and Li, Y. 2003a. *Agrobacterium*-mediated genetic transformation of a *Dendrobium* orchid. Plant Cell, Tissue and Organ Culture 75:63-71.

Men, S., Ming, X., Wang, Y., Liu, R., Wei, C., and Li, Y. 2003b. Genetic transformation of two species of orchid by biolistic bombardment. Plant Cell Reports 21:592-598.

Miki, B., and McHugh, S. 2004. Selectable marker genes in transgenic plants: Applications, alternatives and biosafety. Journal of Biotechnology 107:193-232.

Mishiba, K., Chin, D.P., and Mii, M. 2005. *Agrobacterium*-mediated transformation of *Phalaenopsis* by targeting protocorms at an early stage after germination. Plant Cell Reports 24:297-303.

Mlotshwa, S., Pruss, G.J., and Vance, V. 2008. Small RNAs in viral infection and host defense. Trends in Plant Science 13:375-382.

Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D., and Baulcombe, D. 2009. Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. The Plant journal : for cell and molecular biology 58:165-174.

Namba, R., and Ishii, M. 1971. Failure of aphids to transmit the ORSV and CymMV to orchid plantlets derived from meristem cultures. Phytopathology 61:582-583.

Nan, G., and Kuehnle, A. 1995a. Factors affecting gene delivery by particle bombardment of *Dendrobium* orchids. In Vitro Cellular & Developmental Biology - Plant 31:131-136.

Nan, G., and Kuehnle, A. 1995b. Genetic transformation in *Dendrobium* (orchid). Pages 145-155 in: Plant Protoplasts and Genetic Engineering VI, Biotechnology in Agriculture and Forestry, Y. Bajaj, ed. Springer-Verlag, Berlin.

Nan, G., Tang, C., Kuehnle, A., and Kado, C. 1997. *Dendrobium* orchids contain an inducer of *Agrobacterium* virulence genes. Physiological and Molecular Plant Pathology 51:391-399.

Nan, G., Tang, C., Kuehnle, A., and Kado, C. 1998. Transgenic *Dendrobium* orchid through *Agrobacterium*-mediated transformation. Malayan Orchid Review 32:93-96.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312:436-439.

Nejidat, A., and Beachy, R.N. 1990. Transgenic tobacco plants expressing a tobacco virus coat protein gene are resistant to some tobamoviruses. Molecular Plant-Microbe Interaction 3:247-251.

Nelson, R., Abel, P., and Beachy, R.N. 1987. Lesions and virus accumulation in inoculated transgenic tobacco plants expressing the coat protein gene of Tobacco mosaic virus. Virology 158:126-132.

Nimchuk, Z., Eulgem, T., Holt, B., and Dangl, J. 2003. Recognition and rsponse in the plant immune system. Annual Review of Genetics 37:579-609.

Niu, Q., Lin, S., Reyes, J.L., Chen, K.C., Wu, H.W., Yeh, S.D., and Chua, N.H. 2006. Expression of artificial microRNAs in transgenic Arabidopsis thaliana confers virus resistance. Nature Biotechnology 24:1420-1428.

Olovnikov, I., Aravin, A., and Fejes Toth, K. 2012. Small RNA in the nucleus: the RNA-chromatin ping-pong. Current Opinion in Genetics & Development 22:164-171.

Ossowski, S., Schwab, R., and Weigel, D. 2008. Gene silencing in plants using artificial microRNAs and other small RNAs. The Plant journal : for cell and molecular biology 53:674-690.

Park, S., Yeung, E., Chakrabarty, D., and Paek, K. 2002. An efficient direct induction of protocorm-like bodies from leaf subepidermal cells of *Doritaenopsis* hybrid using thin section culture. Plant Cell Reports 21:46-51.

Park, W., Zhai, J., and Lee, J.Y. 2009. Highly efficient gene silencing using perfect complementary artificial miRNA targeting AP1 or heteromeric artificial miRNA targeting AP1 and CAL genes. Plant Cell Reports 28:469-480.

Park, Y., Kakuta, S., Kano, A., and Okabe, M. 1996. Efficient propagation of protocorm-like bodies of *Phalaenopsis* in liquid medium. Plant Cell, Tissue and Organ Culture 45:79-85.

Pearson, M., and Cole, J. 1991. Further observations on the effects of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* on the growth of *Cymbidium* orchids. Journal of Pathology 131:193-198.

Pfeffer, S., Zavolan, M., Grasser, F., Chien, M., Russo, J., Ju, J., John, B., Enright, A., Marks, D., Sander, C., and Tusch, T. 2004. Identification of virus-encoded microRNAs. Science 304:734-736.

Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M., and Tepfer, M. 2008. Strategies for antiviral resistance in transgenic plants. Molecular Plant Pathology 9:73-83.

Qu, J., Ye, J., and Fang, R. 2007. Artificial microRNA-mediated virus resistance in plants. Journal of Virology 81:6690-6699.

Register, J., and Beachy, R.N. 1988. Resistance to TMV in transgenic plants results from interference with an early event in infection. Virology 66:524-532.

Ryu, K., and Park, W. 1995. The complete nucleotide sequence and genome organisation of odontoglossum ringspot tobamovirus RNA. Archives of Virology 140:1577-1587.

Sablok, G., Pérez-Quintero, A., Hassan, M., Tatarinova, T.V., and Lopez, C. 2011. Artificial microRNAs (amiRNAs) engineering - On how microRNA-based silencing methods have affect current plant silencing research. Biochemical and Biophysical Research Communications 406:315-319.

Sanford, J., Smith, F., and Russell, J. 1993. Optimizing the biolistic process for different biological applications. Methods in Enzymology 217:483-509.

Saxena, P., Hsieh, Y., Alvarado, V., Sainsbury, F., Saunders, K., Lomonossoff, G., and Scholthof, H. 2011. Improved foreign gene expression in plants using a virus-encoded suppressor of RNA silencing modified to be developmentally harmless. Plant Biotechnology Journal 9:703-712.

Schwab, R., Ossowski, S., Warthmann, N., and Weigel, D. 2010. Directed gene silencing with artificial microRNAs. Methods in Molecular Biology 592:71.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D. 2006. Highly specific gene silencing by artificial microRNAs in Arabidopsis. The Plant Cell 18:1121-1133.

Sillivan, C., and Ganem, D. 2005. MicroRNAs and viral infection. Molecular Cell 20:3-7.

Sim, G. (1996). Studies on the physiological status and the role of cytokinin in the flowering of *Dendrobium* orchids (Singapore: National University of Singapore).

Simons, S., and Meyers, B.C. 2011. Small RNA-mediated epigenetic modifications in plants. Current Opinion in Plant Biology 14:148-155.

Sjahril, R., and Mii, M. 2006. High-efficiency *Agrobacterium*-mediated transformation of *Phalaenopsis* using meropenem, a novel antibiotic to eliminate *Agrobacterium*. Journal of Horticulture Science & Biotechnology 81:458-464.

Sjahril, R., Chin, D.P., Khan, R., Yamamura, S., Nakamura, I., Amemiya, Y., and Mii, M. 2006. Transgenic *Phalaenopsis* plants with resistance to Erwinia carotovora produced by introducing wasabi defensin gene using *Agrobacterium* method. Plant Biotechnology 23:191-194.

Soon, T. 1982. A Joy Forever. Vanda Miss Joaquim: Singapore's National Flower. Times Books International, Singapore.

Stark, D., and Beachy, R.N. 1989. Protection against potyvirus infection in transgenic plants: Evidence for broad spectrum resistance. Nature Biotechnolgy 7:1257-1262.

Su, V., and Hsu, B. 2003. Cloning and expression of a putative cytochrome P450 gene that influences the colour of *Phalaenopsis* flowers. Biotechnology Letters 25:1933-1939.

Sullivan, C.M., Grundhoff, A., Tevethia, S., Pipas, J., and Ganem, D. 2005. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. Nature 435:682-686.

Sunkar, R., Chinnusamy, V., and Zhu, J. 2007. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. Trends in Plant Science 12:301-309.

Suwanaketchanatit, C., Piluek, J., Peyachoknagul, S., and Huehne, P. 2007. High efficiency of stable genetic transformation in *Dendrobium* via microprojectile bombardment. Biologia Plantarum 51:720-727.

Tanaka, Y., Y, K., F, B., and J, M. 2005a. Genetic engineering in floriculture. Plant Cell, Tissue and Organ Culture 80:1-24.

Tanaka, Y., Y, K., Bruglier, F., and Mason, J. 2005b. Genetic engineering in floriculture. Plant Cell, Tissue and Organ Culture 79:1-18.

Tee, C., and Maziah, M. 2005. Optimization of biolistic bombardment parameters for *Dendrobium* Sonia 17 calluses using GFP and GUS as the reporter system. Plant Cell, Tissue and Organ Culture 80:77-89.

Tee, C., Marizah, M., Tan, C., and Abdullah, M. 2003. Evaluation of different promoters driving the GFP reporter gene and selected target tissues for particle bombardment of *Dendrobium* Sonia 17. Plant Cell Reports 21:452-458.

Teixeira da Silva, J.A. 2004. Ornamental chrysanthemums: Improvement by biotechnology. Plant Cell, Tissue and Organ Culture 79:1-18.

Tian, L. 2006. Markers gene removal from transgenic plants. Pages 26-29 in: Ornamental and Plant Biotechnology, J.A. Teixeira da Silva, ed. Global Science Books, London.

Tingay, S., McElroy, D., Kalla, R., Fieg, S., Wang, M., Thornton, S., and Brettell, R. 1997. *Agrobacterium tumefaciens*-mediated barley transformation. The Plant journal : for cell and molecular biology 11:1369-1376.

Tumer, N., O'Connell, K., Nelson, R., Sanders, P., Beachy, R.N., Fraley, R., and Shah, D. 1987. Expression of Alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. The EMBO Journal 6:81-88.

Turner, D., Joyce, L., and Butler, P. 1988. The tobacco mosaic virus assembly origin RNA: Functional characteristics defined by directed mutagenesis. Journal of Molecular Biology 203:531-547.

Usami, S., Morikawa, S., Takebe, I., and Machida, Y. 1987. Absence in monocotyledonous plants of the diffusible plant factors inducing T-DNA circularization and vir gene expression in *Agrobacterium*. Molecular and General Genetics 209:221-226.

Vain, P. 2006. Global trends in plant transgenic science and technology (1973-2003). Trends in Biotechnology 24:206-211.

Van Dun, C., and Bol, J. 1988. Transgenic tobacco plants accumulating Tobacco rattle virus coat protein resist infection with Tobacco rattle virus and Pea early browning virus. Virology 167:649-652.

van Dun, C., Bol, J., and van Vloten-Doting, L. 1987. Expression of Alfalfa mosaic virus and Tobacco rattle virus protein genes in transgenic tobacco plants. Virology 159:299-305.

Van Dun, C., Overduin, B., Van Vloten-Doting, L., and Bol, J. 1988. Transgenic tobacco expressing Tobacco streak virus or mutated Alfalfa mosaic virus coat protein does not cross-protect against Alfalfa mosaic virus infection. Virology 164:383-389.

van Ex, F., Jacob, Y., and Martienseen, R. 2011. Multiple roles for small RNAs during plant reproduction. Current Opinion in Plant Biology 14:588-593.

Vanlent, J., Storms, M., Vandermeer, F., Wellink, J., and Goldbach, R. 1991. Tubular structures involved in movement of Cowpea mosaic virus are

also formed in infected cowpea protoplasts. The Journal of General Virology 72:2615-2623.

Vargason, J., Szittya, G., Burgyán, J., and Hall, T. 2003. Size selective recognition of siRNA by an RNA silencing suppressor. Cell 115:799-811.

Walkey, D. 1991. Applied Plant Virology. Springer, Netherlands.

Waterhouse, P.M., Graham, M., and Wang, M. 1998. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proceedings of the National Academy of Sciences of the United States of America 95:13959-13964.

Webster, R., and Granoff, A. (1994). Tobamoviruses. In Encyclopedia of Virology (New York: Academic Press, Harcourt Brace & Company Publishers), pp. 1436-1441.

Whitham, S., Yang, C., and Goodin, M. 2006. Global impact: elucidating plant responses to viral infection. Molecular Plant-Microbe Interaction 19:1207-1215.

Wisler, G., Zettler, F., and Sheehan, T. 1986. Common questions and misconceptions concerning orchid viruses. American Orchid Society Bulletin 55:472-479.

Wolf, S., Deom, C., Beachy, R., and Lucas, W. 1989. Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. Science 246:377-379.

Wolf, S., Deom, C., Beachy, R.N., and Lucas, W.J. 1991. Plasmodesmatal function is probed using transgenic tobacco plants that express a virus movement protein. The Plant Cell 3:593-604.

Wong, S., Chng, C., Lee, Y., Tan, Y., and Zettler, F. 1994. Incidence of cymbidium mosaic and odontoglossum ringspot viruses and their significance in orchid cultivation in Singapore. Crop Protection 13:235-239.

Wong, S., Mahtani, P., Lee, K., Yu, H., Tan, Y., Neo, K., Chan, Y., Wu, M., and Chng, C. 1997. Cymbidium mosaic potexvirus RNA: complete nucleotide sequence and phylogenetic analysis. Archives of Virology 142:383-391.

Wu, Q., Wang, X., and Ding, S. 2010. Viral suppressors of RNA-based viral immunity: host targets. Cell Host & Microbe 8:12-15.

Xie, Z., Kasschan, K., and Carrington, J.C. 2003. Negative feedback regulation of Dicer-Like1 in Arabidopsis by microRNA-guided mRNA degradation. Current Biology 13:784-789.

Xu, P., Zhang, Y., Kang, L., Roossinck, M., and Mysore, K. 2006. Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. Plant Physiology 142:429-440.

Yang, J., Lee, H., Shin, D., Oh, S., Seon, J., Paek, K., and Han, K. 1999. Genetic transformation of *Cymbidium* orchid by particle bombardment. Plant Cell Reports 18:978-984.

Ye, G., Daniell, H., and Sanford, J. 1990. Optimization of delivery of foreign DNA into higher plant chloroplasts. Plant Molecular Biology 15:809-819.

Ye, K., Malinina, L., and Patel, D. 2003. Recognition of small interfering RNA by a viral suppressor of RNA silencing. Nature 426:874-878.

Yee, N., Abdullah, J., Mahmood, M., and Basiron, N. 2008. Co-transfer of gfp, CHS and hptII genes into *Oncidium* Sharry Baby PLB using the biolistic gun. Afr. Journal of Biotechnology 7:2605-2617.

You, S.J., Liau, C.H., Huang, H., Feng, T., Prasad, V., Hsiao, H.H., Lu, J.C., and Chan, M.T. 2003. Sweet pepper ferredoxin-like protein (pflp) gene as a novel selection marker for orchid transformation. Planta 217:60-65.

Yu, B., and Wang, H. 2010. Translational inhibition by micrornas in plants. Pages 41-57 in: MiRNA Regulation of the Translational Machinery, R. Rhoads, ed. Springer, Berlin Heidelberg.

Yu, H., and Xu, Y. 2007. Orchids. Pages 273-288 in: Transgenic Crops VI, E.C. Pua and M.R. Davey, eds. Springer Berlin Heidelberg.

Yu, H., Yang, S.H., and Goh, C.J. 2001. *Agrobacterium*-mediated transformation of a *Dendrobium* orchid with the class 1 knox gene DOH1. Plant Cell Reports 20:301-305.

Yu, Z., Chen, M., Nie, L., Lu, H., Ming, X., Zheng, H., Qu, L., and Chen, Z. 1999. Recovery of transgenic orchid plants with hygromycin selection by particle bombardment to protocorms. Plant Cell, Tissue and Organ Culture 58:87-92.

Zaitlin, M. 1998. The discovery of the casual agent of the Tobacco mosaic disease. Pages 105-110 in: Discoveries in Plant Biology, World Publishing Co., Hong Kong.

Zettler, F., Ko, N., Elliot, M., and Wong, S. 1990. Viruses of orchids and their control. Plant Disease 74:621-626.

Zhang, B., Pan, X., Cannon, C., Cobb, G., and Anderson, T. 2006a. Conservation and divergence of plant microRNA genes. The Plant journal : for cell and molecular biology 46:243-259.

Zhang, X., Yuan, Y., Pei, Y., Lin, S., Tuschl, T., Patel, D., and Chua, N.H. 2006b. Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. Genes & development 20:3255-3268.

Zhao, T., Wang, W., Bai, X., and Qi, Y. 2008. Gene silencing by artificial microRNAs in *Chlamydomonas*. The Plant journal : for cell and molecular biology 58:157-164.